PATTERNS OF MITOSES IN THE GINGIVAL EPITHELIUM OF MICE

A Study by Autoradiography and H^3-Thymidine

by

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C O N T E N T S

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"The power to guess the unseen from the seen, to trace the implications of things, to judge the whole piece by the pattern .....".

The Art of Fiction (1888).

INTRODUCTION

Since the first clinical sign of periodontal disease is concerned with inflammation of the superficial tissue of the periodontium, considerable work has been carried out to determine the normal form and function of the gingivae. Investigations have included clinical and histological observations but in the main these have been static in character, being formulated from histological facts determined by cell form and nuclear or cytoplasmic staining properties.

A dynamic concept of the physiological gingival pocket was introduced by Waerhaug (1952) to which further knowledge has been added by Brill (1962). The first barrier tissue of defence against periodontal disease is the epithelium lining the gingival pocket. Old views of a static epithelium with an organic attachment to the tooth enamel are being discarded and instead, the gingivae are shown to have an active participation in defence of the supporting tissues against organisms of the oral cavity.

With the advent of a cytochemical technique using autoradiography and the radio-isotope tritiated thymidine, which can become incorporated into cell nuclei prior to division, it is now possible to make observations on the two basic functions of cells, namely, mitosis and migration.

The object of this investigation is to re-examine the gingival epithelium of the mouse molar teeth with autoradiography and tritiated thymidine and observe the origin, migration pattern, life cycle and general behaviour of the cells which constitute the tissue.

For this purpose the thesis has been divided into three sections. The first section deals with mitosis of cells and autoradiography; the second with the histogenesis of cells of the gingival epithelium, while the third presents information on the life cycle, migration and behaviour of cells in the mature gingivae around the erupted teeth.

SECTION

I.

SECTION I.

CELL MITOSIS IN TISSUES

Mitosis is defined as indirect cell division. It is the process by which chromosomes become duplicated longitudinally into two or more near equivalent parts which later separate at opposing poles of the cell. (Wilson & Morrison 1961).

Common to almost all cell populations, the study of mitosis in the cells of tissues is used as a measure of tissue growth and tissue activity. From such a study three general types of proliferating cell populations are recognized in the biological systems :-

(i) A population continually increasing in size, such as found in embryonic tissues.

(ii) A steady state renewal cell population in which rate of cell birth equals rate of cell death or loss, e.g., hair follicles, skin, etc.

(iii) A population decreasing in size as found in damaged cell populations where cell death follows mitosis. (Wimber 1963).

REVIEW OF LITERATURE.

Bullough (1962), in a review of mitosis of mammalian tissues, has shown that the control of mitotic activity in an organism is a highly complex mechanism. Numerous factors will influence mitotic rate, such as age, sex, diet, light, temperature, stress, shock and certain specific mitotic stimulants and depressors.

Age: Loeb & Haven (1929a) in guinea pigs and Bullough (1949a) in mice have shown that with increased age the mitotic rate is higher. In mice, the peak increase occurred at middle age, that is, 13 months to 18 months old. Meyer et al (1956) reported similar results in human gingival tissues and showed a significantly higher rate of mitosis in the older age group.

Sex: In guinea pigs, Loeb & Haven (1929b) showed that in female animals, skin mitosis decreased during oestrous and was lower than the figures for male animals of the same age. Bullough (1943) showed a similar variation in mice, viz., an increase in mitosis before and a sudden decrease after ovulation.

Diet: Rabinovitch (1928), in a guinea pig study, produced evidence of lowered mitosis in animals which had been underfed for 10 days. The figures he presented were for thyroid gland epithelium but Loeb et al (1939) demonstrated in guinea pigs a similar decrease in the epidermis of the ear. Bullough (1949b) showed in the mouse epidermis a decrease in mitosis even after as short a period as 36 hours' starvation.

Light: Many investigators have demonstrated variation in rhythm of mitosis to occur throughout the 24 hours of day and night. Carleton (1934) observed the figures on mouse skin. The

maximum was 1.5% between 8.00 p.m. and midnight and his minimum figure of 0.6% occurred at mid-day. The experimental animals, however, were of a variable age, ranging from 8 hours to 7 days after birth. Cooper & Franklin (1940) again investigating mouse epidermis, had maximum mitotic activity at 10.00 a.m. and minimum activity at 10.00 p.m. These investigators showed that the average number of dividing cells during the day-time was double that of the cells dividing at night. Blumenfeld (1939) produced figures from rats which were approximately the same. The maximum mitotic activity was from 8.00 a.m. to 10.00 a.m. Later, this same worker, Blumenfeld (1943), using 2 month old male mice, found that maximum mitotic figures occurred at 12 noon and minimum at 8.00 p.m. Sinha (1956) showed that in day-light, with low body activity, a high number of mitoses was present in rats, while, in darkness, with a high body activity, there was a low mitotic rate in evidence. This cycle he was able to reverse with artificial lighting and darkening conditions and so demonstrated the dependence of the mitotic cycle on light stimulation. The review by Bullough (1962) further contends that, in the healthy animal, all cells are capable of division and that this is part of basic cell function. The apparent stimulus to division seems to be nothing other than the removal of an "inhibitor" substance. Certain hormones possess this attribute, notably those of the gluco-corticoid complex and particularly adrenaline and nor-adrenaline. (Bullough & Lawrence, 1961). In general, it would seem there is an average mitotic rate in normal tissue which has the same

total figure for every 24 hours of adult life.

Tissue Injury: Where a tissue is injured, two factors of importance appear to occur. Firstly, there is a loss of cell contact which leads to cell migration, while, secondly, there is a loss of inhibition of mitosis which allows an abnormally high cell division. In other words, with wounding, the basic actions of cells are allowed freedom. Weiss (1950 : 1958) considers that when a cell loses contact on all sides, it loses its contact "inhibition" and so re-acquires the basic function of motility, whereas, when a cell is damaged, it loses its mitotic inhibition and re-acquires its basic function of mitosis.

Cell damage or tissue injury can occur not only from mechanical or chemical trauma but also as a result of radiation from various substances. (Sparrow (1951) and Swann (1957)). The effects of radiation on cells appear to be complex and are thought to occur at the molecular level. However, the interesting facts lie in the following :-

(i) that light dosage stimulates cells to high mitotic activity;

(ii) that moderate radiation dosage produces damage and prevents cell division;

(iii) that in heavy dosage the vitality of the cell is lost completely. (Bullough, 1962).

More shall be said of this later.

METHODS OF STUDYING CELL MITOSIS

For the study of tissue activity and growth through cell proliferation, three methods are available :-

(i) by calculation of the mitotic index;

(ii) by use of antimitotic drugs;

(iii) by Desoxyribonucleic Acid labelling.

Mitotic Index:

Since mitosis is the one point in the nuclear cycle which can be recognized, the method of examination of a tissue for evidence of cell proliferation was, until recently, by histology and the light microscope. In this, a count of mitotic figures in the cell population was made and data from this observation gave a mitotic index or number of mitoses per 1,000 cells. Results from this method, however, are ineffective in supplying information on time required to replace tissue, as the dividing cell on completion of the division cycle cannot be distinguished from its neighbours.

Antimitotic Drugs:

Use of antimitotic substances, colchicine in particular, introduces a second method in biological research for the investigation of cell mitosis in a tissue. After injection of this substance, cells are trapped in the metaphase stage of their division cycle and thus can be readily recognized. By noting the time interval between injection of colchicine and sacrifice of the animal, information concerning the cell proliferation is of a much more dynamic nature since this technique

allows calculation of the percentage of cells dividing in the tissue during this time interval and thus permits an estimate to be made of the total tissue renewal time. However, there is a disadvantage in the use of colchicine, as cells which have become trapped in metaphase tend to degenerate and disappear from the tissues. (Hooper 1961). Thus, although information is given the investigator on tissue renewal time, it is not possible to trace cell migration.

Desoxyribonucleic Acid Labelling:

Desoxyribonucleic acid or DNA is linked with the chromosomes of the cell nucleus and bears genetic information which is passed, on division of the cell, to the daughter progeny. (Bourne 1964).

As a substance, DNA appears to be metabolically inert and immutable in resting cells. When a cell, however, is preparing to divide, the DNA content is doubled and in the process various precursor nucleotides are taken in for incorporation into the DNA molecule. (Davidson 1960). One of these nucleotides is thymidine which, being specific for the DNA of the cell, can be made an excellent tracer compound for observation of cell mitosis through autoradiography.

It is now accepted that the uptake of radioactive thymidine by cells is indicative of DNA synthesis (Reichard & Estborn 1951 : Friedkin et al 1956) and since it can be taken into the cell without apparent interruption of the normal biology, the technique was adopted for observation of cell mitotic patterns in this investigation of gingival epithelium.

AUTORADIOGRAPHY IN BIOLOGICAL INVESTIGATION

Autoradiography is the method by which photographic materials are used to demonstrate the presence and situation of radioactive substances. As a phenomenon it was first described by Becquerel in 1895 who, when investigating the property of crystal fluorescence, produced an image on a photographic plate, not from crystal fluorescence, but from incorporated radioactive particles within the compound. This led on to his discovery of the radioactive property of uranium.

In giving an account of the history and biological aspects of autoradiography, the review of Gross & Leblond (1947) implies that London in 1904 was the first to make an autoradiograph of animal tissues using the element radium.

To Lacassagne and Lattès (1924) goes the credit for the first refined method of autoradiography. Specimens prepared from rats and rabbits previously injected with polonium were placed under pressure against a photographic film. Good specimen contact with the emulsion, in order to obtain accurate resolution, was thus recognized early in this technique.

In 1946, Bélanger & Leblond removed emulsion from photographic plates, melted it and painted a layer of the molten emulsion on a slide containing the section under investigation. Evans (1947) demonstrated another method in which the histological specimen was floated on top of an unexposed photographic plate. The emulsion was exposed to radioactivity and, later, prior to development of the film, the paraffin wax of the section was dissolved and the

section stained.

Meanwhile, Pelc (1947) suggested a stripping film technique. In this, the photographic emulsion, supported by a gelatin base, was floated in water and picked up on a histology slide in direct contact with the section of the tissue under observation. After suitable exposure time the film was developed and the tissue stained.

Of the three methods outlined, the simplest is that of Bélanger & Leblond (1946) but it is difficult with this technique to procure an even surface of emulsion over the specimen. The Pelc stripping film technique, however, sets no problem in this respect as the emulsion is already prepared as a layer of given thickness. In addition, stripping film is easy to handle with simple darkroom precautions. It was therefore adopted as the autoradiographic technique for this study.

RADIOACTIVE ISOTOPES.

Up until 1934, biological research with autoradiographic techniques was confined to application of naturally occurring radioactive substances, such as radium, (London 1904 : Kotzoreff & Weyl 1923 and Lomholt 1930) and plutonium (Lacassagne & Lattès 1924).

Until the mid-1930's, no attempt was made to introduce to autoradiographic research, trace elements known to be important in normal physiology. Artificially produced radioactivity was discovered in 1934 by Curie and Joliot and from then on, biological work with radio-isotopes steadily increased. Throughout the late 1930's and early 1940's more and more radioactive tracers were developed for tissue metabolism investigations and, at the same time, methods of autoradiography were being improved. (Bélanger & Leblond 1946 : Evans 1947 and Pelc 1947).

In these experiments on metabolism, radio-isotopes of stable elements were offered to an organism for incorporation into some of the tissue compounds. Whether or not they were taken up by the tissue, however, was dependent upon the metabolic state of the tissue at the time. When cells have incorporated a tracer, their movement can be followed by preparing autoradiographs fixed at various time intervals after labelling.

Thus autoradiography not only allows an investigator to determine the location of given compounds within the cells, but, in addition, provides a method of tracing cell movement. The major difficulty in such research is to choose a suitably labelled precursor. For the study of cell proliferation, the subject of this investigation, isotopes are required which are specific for nucleic

acids. Radioactive phosphorus (P^{32}) was one of the first used for this purpose since cells were found to incorporate phosphorus into the desoxyribonucleic acid (DNA) of their nuclei during a part of interphase. However, the P^{32} was also incorporated into cell ribonucleic acid (RNA) and autoradiographs using this isotope to locate DNA were thus rather complicated to produce. (Howard & Pelc 1953). Following the use of radioactive phosphorus, other nucleic acid precursors, such as adenine, cytodine, uridine, deoxycytidine and thymidine, labelled with carbon^{14} and tritium, were applied to cytological research. For the study of cell kinetics, however, the most satisfactory was thymidine.

THYMIDINE.

Although a nucleotide, thymidine is not readily available as a free substance in the body, being formed by a process of methylation of deoxyuridine monophosphate in the presence of tetrahydrofolic acid. (Friedkin & Kornberg 1957). It appears to enter the DNA molecule through thymine at the time of preparation for cell division. (Fig. 1). In this way thymidine becomes a specific component of DNA. (Reichard & Estborn 1951 : Friedkin et al 1956 : Cronkite et al 1959 : Lajtha & Oliver 1959). Since DNA is only present in the cell nuclei (Cantarow & Scheparts 1962) and is doubled by cells as part of the preparation for mitosis (Cronkite et al 1959 : Messier & Leblond 1960), thymidine becomes an excellent medium for mitotic studies.

THYMIDINE A RADIO-ISOTOPE.

Thymidine is usually prepared as a radio-isotope by the attachment of C^{14} or H^{3} to the molecule. Both these isotopes provide radioactivity by the emission of soft beta rays. In the case of C^{14}, the rays have a particle range of 300 microns and an average range of 60 microns. Although this wide range is an advantage in thick section autoradiography, it greatly decreases the efficiency in autoradiographic interpretation. Non-radioactive cell nuclei are difficult to distinguish from the radioactive because of the widespread exposure of the film emulsion. An example of this is seen in Fig. 2 in which cells have incorporated thymidine C^{14} in their nucleus.

TRITIATED THYMIDINE.

Tritium is an active isotope of hydrogen with the unstable

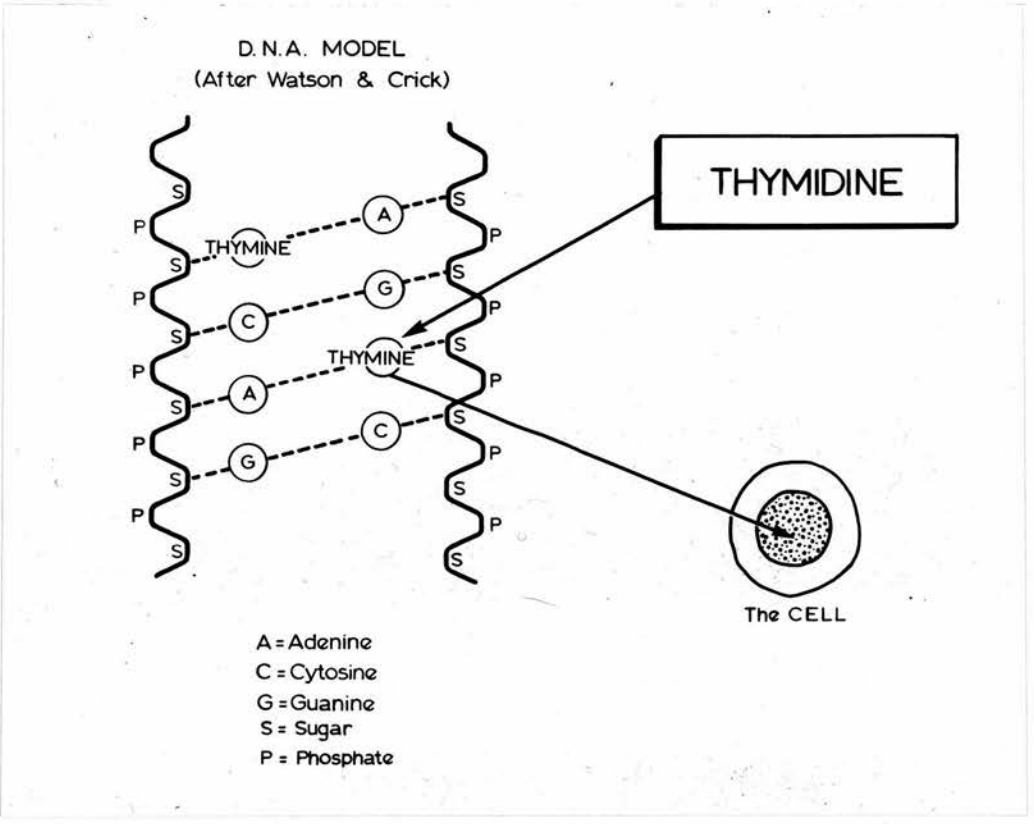

Fig. 1. Diagram illustrating the uptake of H^3-thymidine into a cell nucleus via thymine and desoxyribonucleic acid.

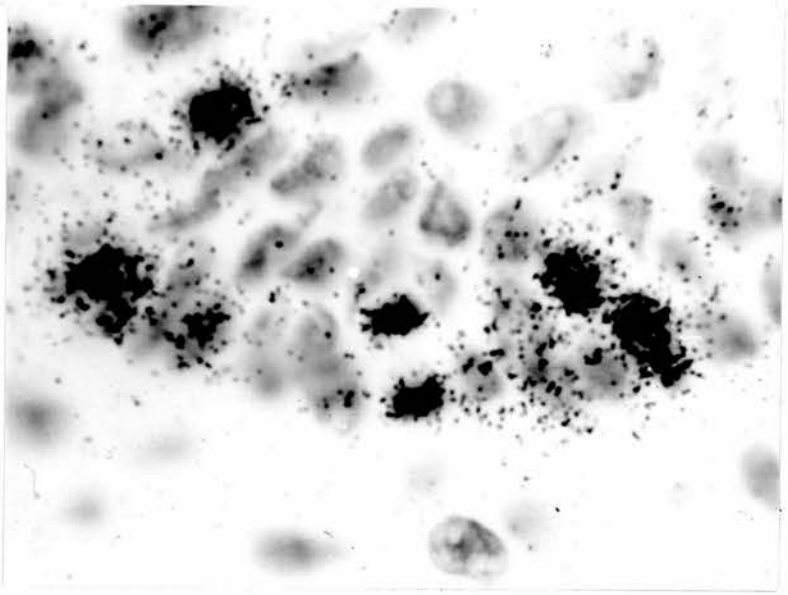

Fig. 2. Autoradiograph of mouse oral epithelium 1 hour after injection of C^{14}-thymidine demonstrating the wide scatter of silver grains around the cells undergoing DNA synthesis.

Stained haematoxylin. Orig. mag. X320.

nuclear combination of two neutrons and one proton which break down to one neutron and two protons of helium. (Fig. 3). (Fitzgerald et al 1953).

The resultant change allows the emission of a beta particle with an average range of 1.5 microns (Lajtha & Oliver 1959). Having a half-life of 12.1± .05 years tritium is, in addition, readily compatible with body fluids (Cronkite et al 1959) and is thus particularly suitable for cytological studies. (Fitzgerald et al 1951).

A significant advance in autoradiographic research was made when Hughes managed to incorporate tritium into thymidine at Brookhaven National Laboratories in 1955 because then, cells incorporating thymidine and thus preparing for division could be accurately assessed. (Fig. 4).

Certain assumptions must be made, however, for the acceptance of this experimental method for investigation of cell populations :-

1. that the progenitor cycle of cells is divided into four phases, as illustrated in Fig. 5;
2. that, in a renewing cell population, if a balance is to be maintained, each mitotic division of a progenitor cell should be followed by the return of one daughter cell to the progenitor pool to replace the cell lost;
3. that incorporation of H^3-thymidine into cell nuclei is indicative of DNA synthesis;
4. that the circulating radio-isotope is only present for a short period of time after injection and does not remain as a pool within the body;

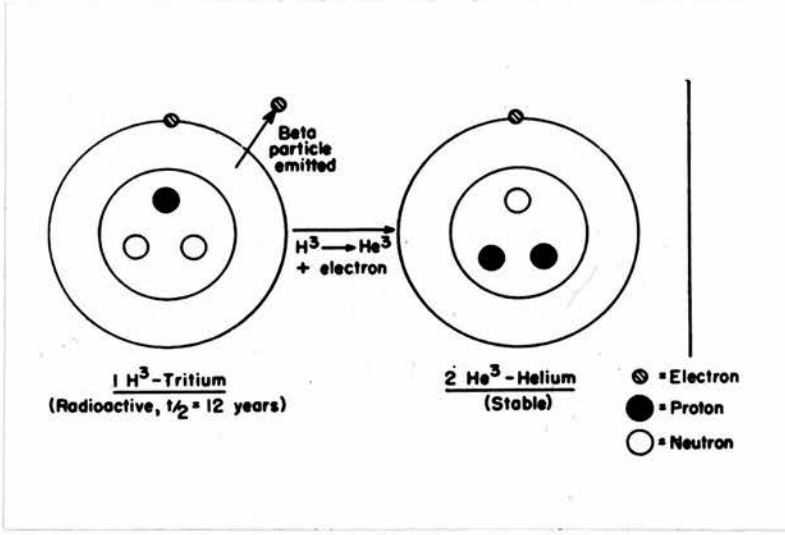

Fig. 3. Diagram illustrating the radioactive decay of Tritium. Being unstable, it breaks down with beta particle emission into Helium.

(After Fitzgerald et al 1953).

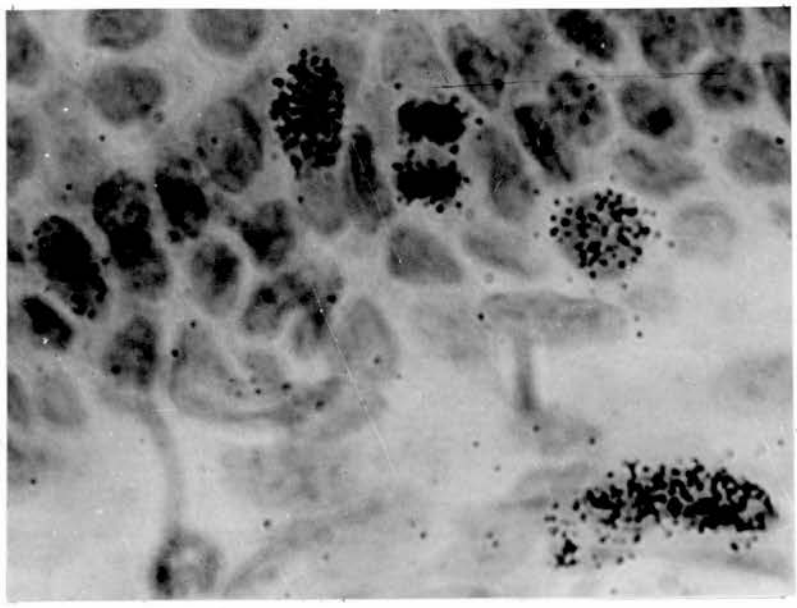

Fig. 4. Autoradiograph of mouse oral epithelium 1 hour after labelling with H^3-thymidine.

Compared with Fig. 2. little "scatter" is evident and labelled cells can be accurately determined.

Stained haematoxylin. Orig. mag. X320.

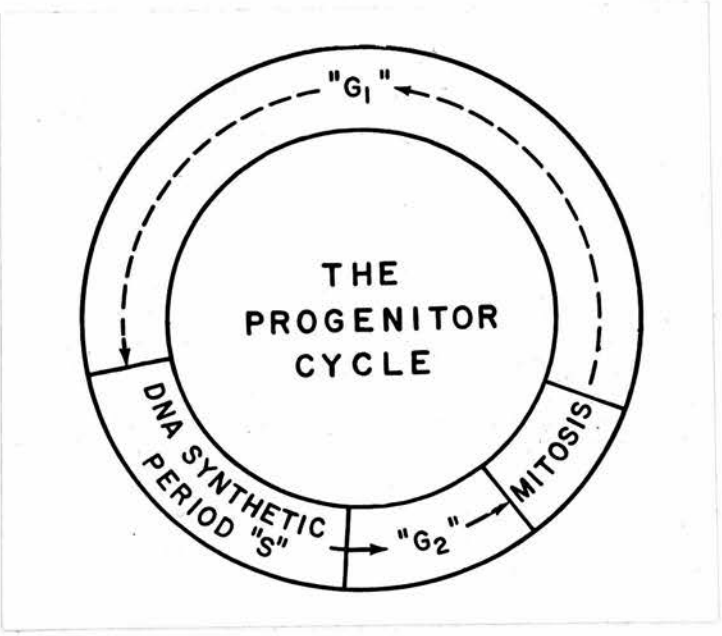

Fig. 5. Diagram illustrating the four phases of the progenitor cycle of cells.

(After Cameron & Greulich 1963).

5. that the cells which have accepted the labelling isotope are not altered biologically. (Cameron & Greulich 1963).

Howard & Pelc (1953) demonstrated in a study of normal and irradiated cells not only the four compartments of the progenitor cycle but also that each cell must pass through these compartments in strict order towards mitosis. Support of this theory has been given by many workers but particularly by the studies of Quastler & Sherman (1959) and Sherman & Quastler (1960).

In cell populations balance between cell loss and cell mitosis must exist to maintain the normal size of the tissue. (Wimber 1963).

The argument that H^3-thymidine is incorporated only into cell nuclei and indicative therefore of DNA synthesis, has already been made and is illustrated in Fig. 1.

The time of blood circulation of the radio-isotope would appear to be dependent upon the mode of administration. In man a rapid fall in the radioactivity was found 1 hour after intravenous injection. (Cronkite et al 1959). After subcutaneous injection, Messier & Leblond (1960) demonstrated a peak concentration in the blood stream of rats after 20 minutes and total clearance from the plasma after 60 minutes. Quastler & Sherman (1959) from grain count autoradiographs found almost a complete uptake of thymidine by the intestinal crypts of mice 16 minutes after intraperitoneal injection.

Skougaard & Stewart (1965) demonstrated in mice, by intramuscular and intraperitoneal injection, an exceedingly rapid uptake of the radio tracer by both routes. They found a peak range of

H^3-thymidine concentration in the plasma within 2-5 minutes. It would seem therefore that the demands of short circulation time with no body pool of isotope are satisfied by all routes of administration but that, as expected, the intravenous, intraperitoneal and intramuscular routes are quicker than the subcutaneous in meeting these requirements.

Only in the fifth assumption is there apparent disagreement amongst workers and this concerns not an actual metabolic alteration of the cells but possible radiation effects to the nucleus of the cell by the tritium beta particles. Because tritium has a short beta particle range, the nucleus of the cell is under constant bombardment from the emission. Thus, the very factor which makes the isotope so accurate for high resolution autoradiography is considered to be its major disadvantage biologically (Lajtha & Oliver 1959). Similar reservations are expressed by Plaut (1959) : Wimber (1959) : Drew & Painter (1959) : Johnson & Cronkite (1959) and Fry & Lesher (1961). However, these views are mainly concerned with long-term effects on cell chromosomes and these are not germane to this study.

Bullough (1962), already discussed in the literature review, showed that the effects of radiation on cell mitosis depended upon dosage and varied from increased mitosis to cell death. Greulich et al (1961), reported finding increased mitosis in intestinal epithelium of the mouse following administration of tritiated thymidine. However, in tissue culture experiments with He La cells, Painter, Drew & Hughes (1958) reported the opposite effect with inhibition of tissue growth. Rubini et al (1962) demonstrated in an

investigation of in vitro metabolism of H^3-thymidine that only very small quantities of tritiated thymidine were incorporated into new DNA and therefore they maintained that the yield of radiation effects was unlikely to be harmful.

Wimber (1963) considered that in an investigation with H^3-thymidine it was important to use a concentration of isotope at a level which would cause no detectable effect on the system being studied, but did not say what this level should be.

Since observations and conclusions in this series of experiments on mouse gingival epithelium are to be conducted following injection of animals with H^3-thymidine in the manner of Messier & Leblond (1960), it was necessary to determine whether the incorporation of the isotope at a level of 1 micro-curie per gram body weight would affect the mitotic rate of the oral epithelial tissues.

PRESENT INVESTIGATION

IN-VIVO EFFECTS OF TRITIATED THYMIDINE ON ORAL EPITHELIAL CELL MITOSIS

To determine the stimulative or depressant effects of tritiated thymidine on the mitotic rate of oral squamous epithelial cells of the mouse, 72 male animals of the same genetic inbred strain and of the same age (25 days $\pm$ 1 day) were selected.

The review of literature has shown that, in addition to age and sex, several other factors will influence mitotic rate in animal tissues, namely, diet, temperature, stress and diurnal rhythm. To reduce error, these factors were all kept constant during the experiment. The animals were divided into three groups of 24 and injected subcutaneously as follows :-

Group 1 - with 0.1 ml. of normal saline

Group 2 - with 0.1 ml. of .01% thymidine in normal saline solution

Group 3 - with 0.1 ml. of H^3-thymidine (specific activity 3 ~~micro~~-curies/milli-mole) in normal saline solution containing 0.073 mg/ml free thymidine.

The average weight of the animals was 10 grams and the dosage of tritiated thymidine administered was of the order of 1 microcurie per gram body weight. As the synthesis phase of mouse epithelial cells was calculated at approximately $8\frac{1}{2}$ hours' duration, (Toto & Ojha 1962), the effects of the test substances were studied within this time interval. A 3 hour interval after injection was arbitrarily chosen for sacrifice so that, even although a stimu-

lation in mitotic rate was present, cells which had accepted the full quantity of injected nucleotide would not have divided within the time limit. All injections were given within 20 minutes. On sacrifice the tongues and palates of each animal were removed and fixed in Carnoy for 6 hours. Distortion shrinkage of the tissue is liable to occur through the acetic acid present in this fixing solution and to minimize this effect, small pieces of tissue were carried through to wax imbedding immediately after this short period of fixation had elapsed. Serial sections were cut at 5 microns and stained by the Feulgen method, using brilliant green as a counter stain. Counts were taken at 800 diameters of the mitotic figures in the basal layer of the palate and the under-surface of the tongue. Only sections showing a clear basal layer of epithelial cells were used for readings. If an oblique cut of the tissue was evident in the sections examined, these were discarded. For each animal two separate counts were made of the mitoses in 1,000 basal cells of both tissues and the index was then recorded as a percentage. The complete data is shown in the appendix.

RESULTS.

The mean percentage and variance of mitotic figures for 24 observations in tongue and palate epithelium of the three groups of animals formed for this investigation are shown in Tables I and II.

TABLE I

Mean and variance of mitotic figures for 24 observations in tongue epithelium for Saline, Thymidine and H^3-thymidine injected animals. (Complete data in appendix).

	Mean ($\bar{x}$)	Variance (s^2)
Group 1 (Saline)	1.08	0.050
Group 2 (Thymidine)	1.32	0.072
Group 3 (H^3-thymidine)	1.15	0.039

TABLE II

Mean and variance of mitotic figures for 24 observations in palate epithelium for Saline, Thymidine and H^3-thymidine injected animals. (Complete date in appendix).

	Mean ($\bar{x}$)	Variance (s^2)
Group 1 (Saline)	0.94	0.093
Group 2 (Thymidine)	1.29	0.085
Group 3 (H^3-thymidine)	1.18	0.082

To compare the difference of means and apply the t-test of significance between these means it was necessary in the first instance to transform the percentage figures by the Arc. Sin. transformation to give the following data :-

TABLE III

Comparison of means of mitotic figures of Palate experiments. Thymidine and H^3-thymidine with Control Group Saline student t-test. Data transformed by Arc. Sin. transformation. (Complete data in appendix).

	Mean $\bar{x}_1$	$\sum x^2$	df	t	P
Saline (Control)	5.48	740.69	23	2.87	0.01
H^3-thymidine	6.17	926.16	23		
Saline (Control)	5.48	740.69	23	4.19	0.001
Thymidine (Plain)	6.49	1018.65	23		

TABLE IV

Comparison of means of mitotic figures of Tongue experiments. Thymidine and H^3-thymidine with Control Group Saline student t-test. Data transformed by Arc. Sin. transformation. (Complete data in appendix).

	Mean $\bar{x}_1$	$\sum x^2$	df	t	P
Saline (Control)	5.91	848.76	23	1.36	0.2
H^3-thymidine	6.14	912.39	23		
Saline (Control)	5.91	848.76	23	3.40	0.001
Thymidine (Plain)	6.56	1044.54	23		

DISCUSSION.

The results of this experiment indicated that in the oral epithelium of mice exogenous thymidine produced an increase in the mitotic rate. The effects were seen in both test tissues, viz., palate and tongue. A highly significant result was given when plain thymidine was injected (Tables III & IV) with an average increase in the percentage of mitoses of 22.2% for tongue and 37.2% for palate epithelium (Tables I & II). With H^3-thymidine an increase in mitoses was again recorded, viz., 6.5% in tongue and 25.5% in palate (Tables I & II). Neither the level nor the significance of the H^3-thymidine results were, however, as great as that experienced in the plain thymidine group (Tables III & IV).

In a similar experiment, Greulich et al (1961) claimed a 29.2% average increase in mitoses of intestinal epithelium following the administration of thymidine and H^3-thymidine. This figure reported by Greulich was, however, calculated without separating the experimental animals into plain thymidine and tritiated thymidine groups. The trend for an increase in mitosis following thymidine and H^3-thymidine injection was, however, supported.

The mechanism of this increase could be explained in several ways :-

Interference with the Inhibitor Mechanism.

The mechanism of mitosis control seemed to be one of inhibition (Bullough 1962) and it may be that thymidine has an effect within the organism to counter the inhibition mechanism.

Toxic Action.

Mitotic poisons, such as colchicine, cause cells to be trapped at the metaphase stage of their division cycle and produce a higher mitotic index. Such an explanation could not be considered valid in H^3-thymidine studies as a feature of autoradiography with this isotope is the lack of interference with the mitotic cycle which allows complete observation of the migration pattern of the cells. (Messier & Leblond 1960). Furthermore, in histological examination of the tissues in this experiment, all stages of nuclear division of cells were recognizable, so toxic activity in the mitotic spindle seems unlikely.

Physiological Action.

Barr (1963) conducted experiments on barley root tips and on He La cells through which to follow both the long term and short term effects of exogenous thymidine on the mitotic cycle. The conclusions of the experiment were, that an initial increase of mitotic rate was evident but that this did not in itself represent an increase in cellular proliferation. Instead, it was shown that the mitotic rate increase was due to an extension of the time of metaphase. This was explained by Friedkin (1959) as occurring from a bypassing of the methylation of deoxyuridine monophosphate, normally necessary in the DNA synthesis phase of the mitotic cycle, because of the presence of exogenous molecules of thymidine. As a result of such a short cut, DNA synthesis was speeded up and a cell so affected proceeded to the subsequent division stages more quickly. However, Friedkin stated that the phenomenon corrected itself after the first division and that no further increase in

cell proliferation was thereafter detected.

Greulich, Cameron & Thrasher (1961) proposed that free thymidine acted as the stimulus to cell mitosis. Thomson, Paul & Davidson (1958) demonstrated the immediate use of any free nucleosides by cell systems and using this argument, Greulich et al (1961) suggested that the free exogenous thymidine, in being utilized for DNA synthesis, was responsible for the spurt in the mitotic cycle.

Rueckert & Mueller (1960) were able to show that DNA synthesis in vitro could be interrupted by use of folic acid antagonists and that this antagonistic action could be counteracted by the addition of thymidine, which not only re-established DNA synthesis but also caused an increase in cell division.

The results of the present investigation would support such an argument since, in both experimental solutions free thymidine was present. There was a reduced stimulation of mitosis in the H^3-thymidine group of animals compared with the plain thymidine group but there was less free thymidine available in the tritiated solution, viz., 0.073 mg./ml. compared with 0.1 mg./ml. in the plain injection of thymidine.

As the resultant stimulation to mitoses following H^3-thymidine injection ranged from 6.5% - 25.5%, it would seem necessary to accept the mean of these figures, namely 16%, as the expected increase in mitoses for mouse oral epithelium. Thus, any mathematical calculations utilizing radioactive indices concerned with initial labelling of cells would have to take this increase into account. Such a correction is particularly pertinent in the light of the findings of Friedkin (1959) that after initial division the

mitotic stimulus was spent.

The experiments throughout Sections II and III are therefore conducted with the same dosage and concentration of isotope with the knowledge that although an increase in mitosis is present the true pattern of mitoses will not be impaired.

CONCLUSIONS.

1. In the oral epithelium of the mouse, represented by tongue and palate, there was an increase in the mitotic index up to 3 hours after the introduction of exogenous thymidine.

2. Following injection of H^3-thymidine at a dosage of 1 micro-curie/gram body weight, oral epithelial cells were not halted at any point of their division cycle as all mitotic configurations were seen and recognized.

3. The range of increased mitosis in the test tissues was less (6.5% - 25.5%) for tritiated thymidine than for plain thymidine (22.2% - 37.2%).

4. In H^3-thymidine autoradiography of mouse oral epithelium, a correction of 16% should be made in the calculation of data utilizing the radioactive indices (% labelled cells) of initial cell labelling.

SECTION

II.

SECTION II.

THE FORMATION OF GINGIVAL EPITHELIUM

An investigation of the origin and formation of the gingival epithelium must, of necessity, consider the histological changes seen in the oral and enamel epithelial tissues which unite to form the mature gingivae around the teeth. That histological changes do occur in both tissues is not in dispute and many authors have described them, notably Hunter (1803), Tomes (1848), Waldeyer (1871), Paul (1896), Williams (1896), James (1909), Gottlieb (1921), Orban (1928), Saunders et al (1942), Orban et al (1943), Nuckolls et al (1943) : (1947), Marsland (1951) : (1952), Johnson & Bevelander (1957), McHugh (1959) : (1961), Uohara (1959), Hunt (1959) and Hunt & Paynter (1963).

There is, however, disagreement as to which part of the original enamel organ is associated with gingival formation. It is necessary therefore, in tracing the histogenesis, to observe the changes that occur in this organ during and following enamel formation.

The enamel organ is composed of four separate layers of cells which are described and named according to their morphology, function or location :-

(i) The outer enamel epithelium.

A single cell layer of cuboidal cells separated from the dental sac by a definite but thin basement membrane.

(ii) The stellate reticulum.

Adjacent to the outer enamel epithelium this layer is several cells thick. Each cell is star shaped and well separated from its neighbour by an abundance of inter-cellular substance.

(iii) The ameloblasts.

These cells constitute the single cell layer in closest proximity to the dental papilla. They have a columnar morphology with their nuclei terminally placed.

(iv) The stratum intermedium.

Cells of this layer are situated between the ameloblasts and the stellate reticulum. Flat or cuboidal in shape these cells form a layer one to three cells deep. (Sicher 1962).

In the mouse the enamel organ is best seen in the continually growing incisor and is illustrated in Fig. 6.

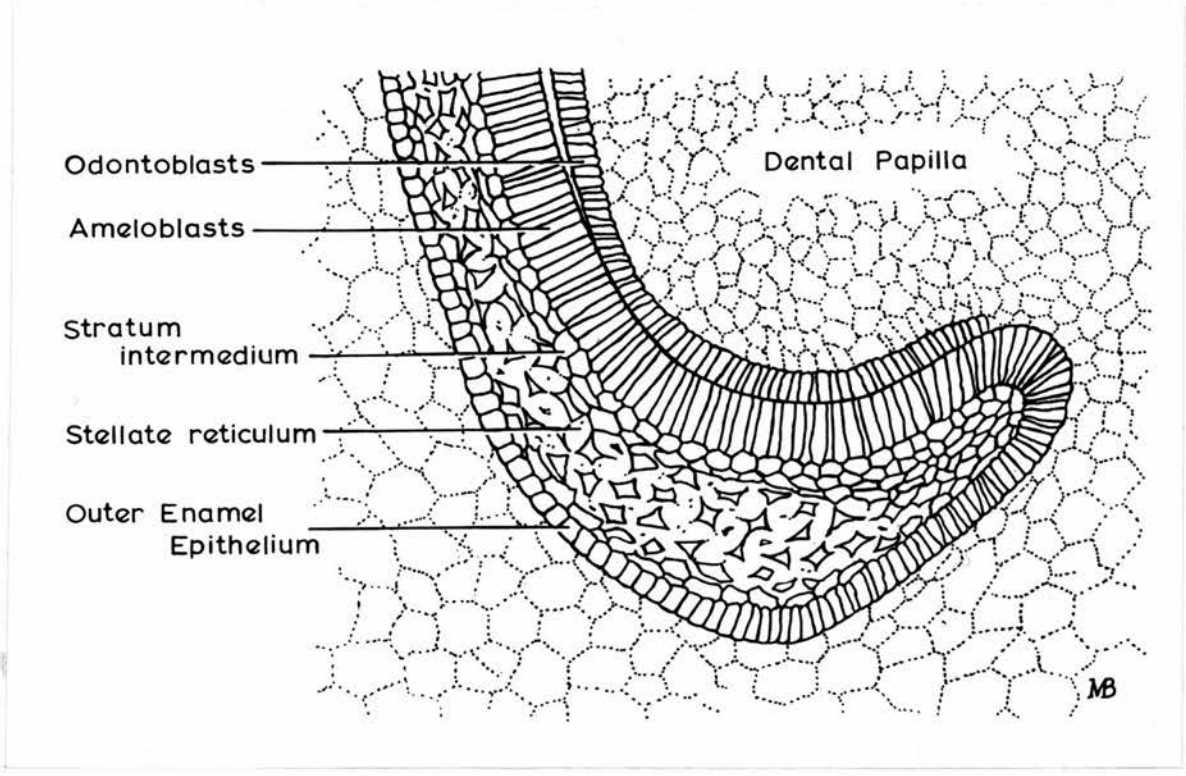

Fig. 6. Diagram of the enamel organ of the mouse molar tooth illustrating the individual cell layers.

REVIEW OF LITERATURE.

According to Marsland (1951), the start of enamel matrix formation was accompanied by collapse of the stellate reticulum and, of the original four layers of cells only the ameloblasts and stratum intermedium were then readily recognizable. Completion of matrix formation and enamel maturation showed further changes in the enamel epithelium, as described by Marsland (1952). Demonstrated in the rat, they were first observed over the cusps of the first molar of a 16 day old animal, that is, two days before the tooth erupted. Initially the change consisted of a disappearance of globules in the ameloblasts followed by a morphological alteration to produce what Marsland described as "short ameloblasts". The cells of the stratum intermedium showed a reversion to a more basal form of cell with characteristics similar to those of the outer enamel epithelium. These cells took on a squamous form and resembled the prickle cells of the oral epithelium. As the tooth erupted this epithelium united with the oral epithelium. From electron microscopy of human teeth, Ussing (1955) demonstrated an ameloblast layer, a stratum intermedium and a papillary layer which was claimed to be derived from cells of the outer enamel epithelium.

McHugh (1959) claimed that, following enamel maturation in the hamster, the rat, the monkey and man, the enamel organ was reduced to three cell layers - the ameloblasts, stratum intermedium and an outer layer formed by fusion of the stellate reticulum and the outer enamel epithelium. He went on to postulate that this outer layer was responsible for the initial proliferation in the formation

of gingival epithelium. Adloff (1924) questioned the presence of an outer enamel epithelial layer at the time of tooth eruption and considered that only an ameloblast layer and a stratum intermedium layer were present. Thomas (1925) showed that remnants of ameloblasts and a recognizable stratum intermedium were still located at the enamel surface when the tooth crown was erupting through and uniting with the oral epithelium.

Observations on the pig molar by Johnson & Bevelander (1957), on the guinea pig molar by Hunt (1959) and on the rat molar by Uohara (1959) suggested, by histological methods, that the cells of the stratum intermedium constituted the proliferative layer of the reduced enamel epithelium responsible for formation of gingival epithelium. Cohen (1959), however, in considering epithelial changes in the gingival papilla of the rat, pointed out that two distinct epithelial cell layers were present - the oral epithelium which he referred to as a proliferating tissue, and the enamel epithelium which he considered had no potential for expansion or proliferation and constituted "a degenerating tissue doomed to extinction". Furthermore, he considered that this enamel epithelium was not in any way an actively growing tissue.

Although there is little disagreement as to the part played by oral epithelium, from the foregoing review of the literature it can be seen that there is considerable divergence of opinion regarding which layer of the enamel organ is associated with the histogenesis of gingival epithelium. The hypotheses outlined made reference to the proliferation and non-proliferation of certain cell groups of the reduced enamel epithelium. As pro-

liferation of a tissue can only occur by mitoses of cells constituting that tissue, the application of tritiated thymidine autoradiography would seem an excellent technique for studying the origin and behaviour of cells taking part in the formation of gingival epithelium through an investigation of the mitotic pattern.

PRESENT INVESTIGATION

TRITIATED THYMIDINE AUTORADIOGRAPHY OF EPITHELIUM RELATED TO THE ERUPTING MOUSE MOLAR TEETH

Because of its size, the mouse is an excellent animal for studies with experimental isotopes. Although the incisor teeth of the mouse will show some of the early changes associated with the histogenesis of gingival epithelium, only the erupting molar teeth can present the full data necessary for this present investigation.

A full account of the eruption times of the mouse molar teeth has been given by Mahn (1890) in which he states that the 1st molar erupts at 16 days, the 2nd molar at 22 days and the 3rd molar at 27-28 days after birth.

EXPERIMENTAL METHOD.

18 male animals from the same inbred strain (J.U. stock of the Animal Genetics Department, University of Edinburgh) were used for the experiment and they were subjected to identical cage and feeding conditions throughout. The mice were separated into three groups of six, according to age :-

Group I - 25 days old

Group II - 26 days old

Group III - 27 days old.

Since Mahn (1890) claimed that eruption of mouse molar teeth was an exceedingly rapid process, it was felt that the selected age range of the three groups of animals would provide enough latitude for complete autoradiographic observations on the eruption changes over the 3rd molar teeth.

Injections of H^3-thymidine (specific activity 3 curies/millimole) were given subcutaneously at a dosage of 1 micro-curie per gram body weight. Following this, in each group, the animals were sacrificed at intervals of 1 hour, 4 hours, 12 hours, 24 hours, 2 days and 3 days. At sacrifice, the tissues were perfused with 10% neutral formalin via the right atrium of the heart, after which the heads were removed and fixed in a similar solution. Decalcification was carried out at room temperature with a 5% solution of ethylenediamine tetra-acetic acid until X-ray evidence showed that the process was complete. The tissues were then double embedded in celloidin and paraffin wax and sections were cut sagittally through all teeth and coronally through the 3rd molar teeth of both jaws at a thickness of 5 microns. Autoradiographs were prepared with Kodak AR.10 stripping film, according to the method of Pelc (1947) and stored at a temperature of $5^{\circ}C$ in light tight boxes for 21 days. Every fifth section was kept for histological study by light microscopy. Staining of the autoradiographs was carried out with a dilute solution of haematoxylin while the histological sections were stained by the Papanicolaou method or haematoxylin and eosin.

RESULTS.

ENAMEL EPITHELIUM.

Observations on enamel epithelium were recorded both histologically and autoradiographically in relation to the 3 stages of the enamel epithelial function :-

Pre-enamel matrix formation.

Post-enamel matrix formation and pre-enamel maturation.

Post-enamel maturation.

Only by study of the autoradiographs of these stages throughout the various time intervals after injection of the tracing isotope can the complete cell mitosis and migration pattern be followed.

Pre-enamel Matrix Formation.

At this stage the 4 layers of cells described previously in Fig. 6 were readily recognized histologically in all groups of animals. At the 1 hour interval, after injection of H^3-thymidine, pre-ameloblast cells, the adjacent stratum intermedium and the outer enamel epithelium, carried tritium nuclear labelling but the cells of the stellate reticulum at this interval did not contain the isotope. This was best seen in the continuously growing incisor tooth. (Figs. 7 & 8).

Observations of this area, however, 12 hours after injection of the isotope, showed a labelling of cells of the stellate reticulum in addition to the ameloblasts, stratum intermedium and outer enamel epithelium. (Fig. 9). This was in contrast to the findings of the 1 hour specimens. A

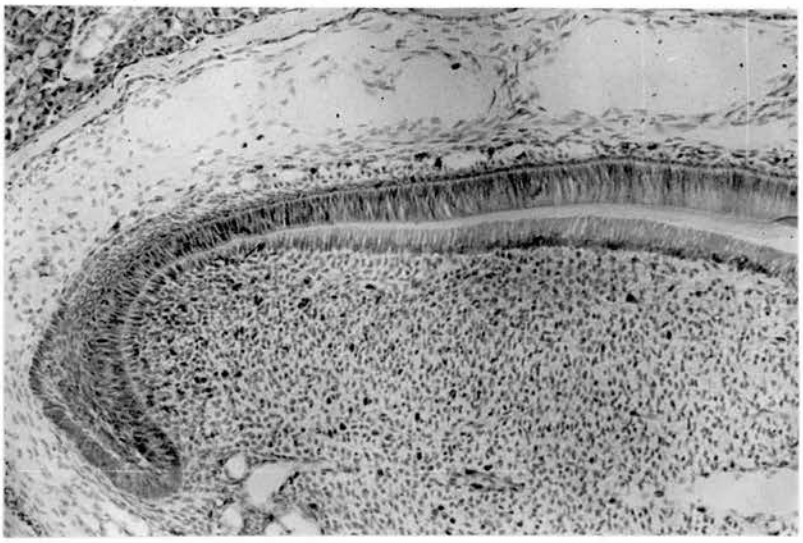

Fig. 7. Autoradiograph of the cervical loop of the developing incisor of a mouse 1 hour after injection of H^3-thymidine.

Stained haematoxylin. Orig. mag. X80.

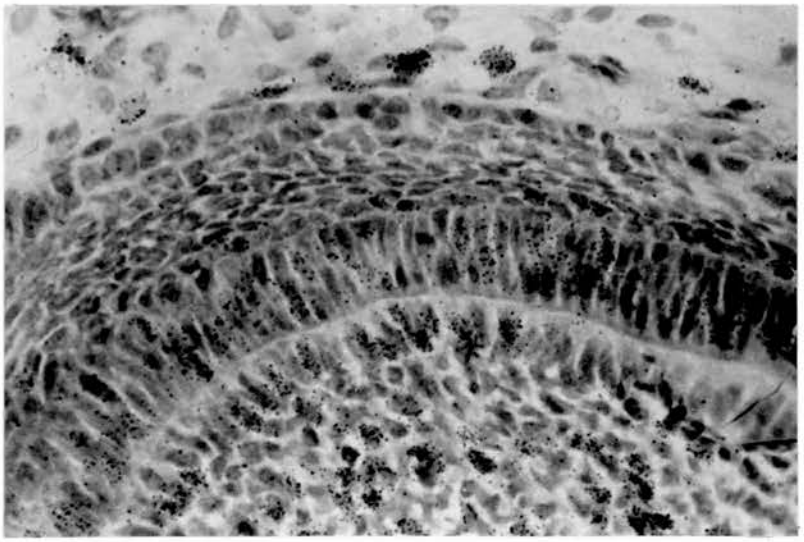

Fig. 8. 1 hour autoradiograph of the cervical loop of the incisor tooth in Fig. 7. showing labelling in the pre-ameloblasts and stratum intermedium. Note in the stratum intermedium the presence of an unlabelled mitotic figure in telophase.

Stained haematoxylin. Orig. mag. X320.

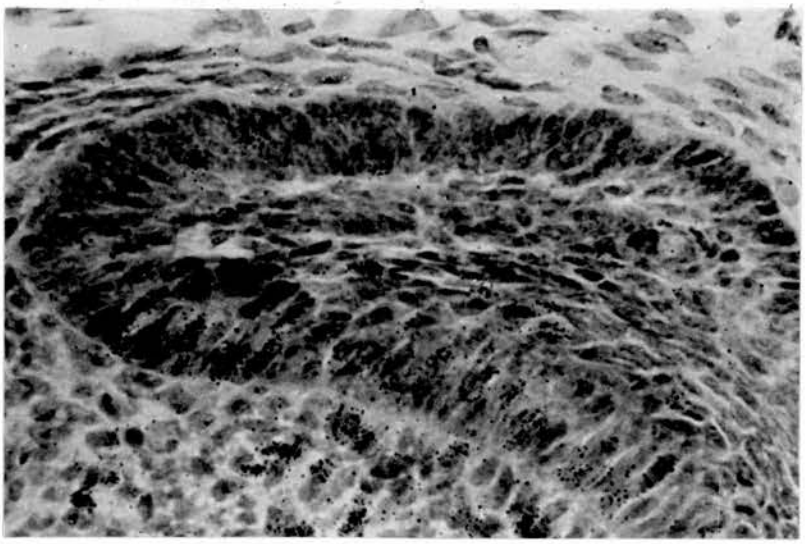

Fig. 9. Autoradiograph of the cervical loop of a developing incisor 12 hours after injection of H^3-thymidine. Labelled cells now present throughout the enamel organ. A mitotic figure is seen in the stratum intermedium.

Stained haematoxylin. Orig. mag. X320.

dilution of labelling intensity compatible with cell division from the stratum intermedium had occurred between the 1 hour and 12 hour period. Cells of the stellate reticulum next to the obvious stratum intermedium had a similar grain count to those of the stratum intermedium. By 24 hours, 48 hours and 72 hours after injection, labelling was apparent deeper in the stellate reticulum and even greater dilution of labelling in the stratum intermedium had taken place.

Post-enamel Matrix Formation and Pre-enamel Maturation.

At this stage, only two layers of cells of the original enamel organ were seen histologically, viz., the stratum intermedium and the ameloblast layer. The stellate reticulum had collapsed and the outer enamel epithelial layer was not recognizable. At the 1 hour interval, after injection of tritiated thymidine, autoradiographs failed to demonstrate any of the epithelial cells of the collapsed enamel organ with DNA labelling (Fig. 10), the only labelling observed at this time being in the connective tissue now placed in close proximity to the stratum intermedium. Endothelial cells of the many blood vessels observed within this connective tissue were similarly labelled. At the remaining time intervals dilution of labelling had occurred in these cells but the labelling distribution remained the same.

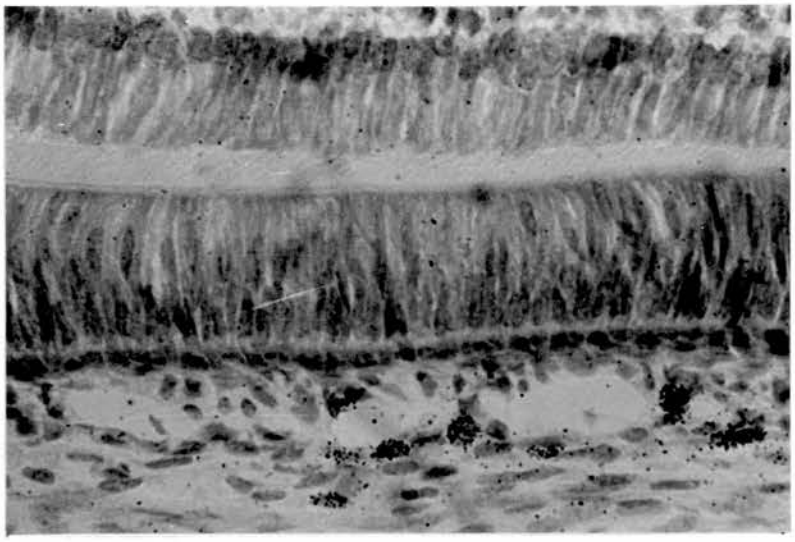

Fig. 10. 1 hour after injection of H^3-thymidine, an autoradiograph of the reduced enamel epithelium of a mouse incisor. At this stage, prior to enamel maturation, no labelling of the reduced enamel epithelium is found. Instead, labelled cells are confined to the endothelial cells of the blood vessels and connective tissue.

Stained haematoxylin. Orig. mag. X320.

Post-Enamel Maturation.

Histologically, the changes observed in the reduced enamel epithelium over the 3rd molar teeth were shortening of the ameloblasts and loss of organic enamel matrix with the decalcification procedure. In all animal groups where the enamel epithelium was found beneath the alveolar bone crest, the stratum intermedium consisted of a single cell layer.

Where, in Group I animals, tooth eruption had carried the reduced enamel epithelium above the crest of the alveolus, there was a 3-5 cell increase in the layer external to the shortened ameloblasts. As rounded cells with rounded nuclei, their morphology and staining characteristics were identical to the cells of the stratum intermedium. These characteristics were well demonstrated with haematoxylin and eosin, and also with the Papanicolaou staining method. (Figs. 11 & 11a).

1 hour autoradiographs demonstrated labelling in the single cell stratum intermedium but none was seen in the ameloblasts. (Figs. 12 & 12a). Where the cell layer had expanded, labelling was present in "isolated islets" and at variable cell distance from the ameloblasts. (Fig. 12b). These islets did not appear to be related to the overlying oral epithelium which, by contrast, had a well defined basal layer containing labelled cells.

In Group II animals the isolated islets of cells over the 3rd molar crown had coalesced, lost the rounded cell morphology and become flattened squamous cells. 1 hour autoradiographs of this area demonstrated labelled cells at various levels throughout the layer. These ranged from cells adjacent to the ameloblasts, which

Fig. 11.

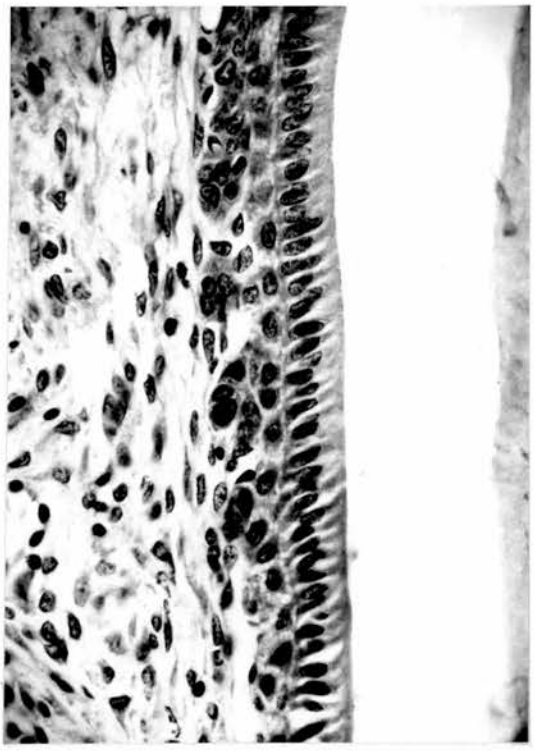

Fig. 11a.

Fig. 11. Photomicrograph of an erupting mouse molar tooth showing oral and reduced enamel epithelium. Above the alveolar crest the enamel epithelium is several cells deep while beneath this point it consists of ameloblasts and a single cell layer of stratum intermedium.

Stained Papanicolaou. Orig. mag. X80.

Fig. 11a. Higher power photomicrograph of the section in Fig. 11., showing the single cell stratum intermedium and proliferating cell groups.

Stained Papanicolaou. Orig. mag. X320.

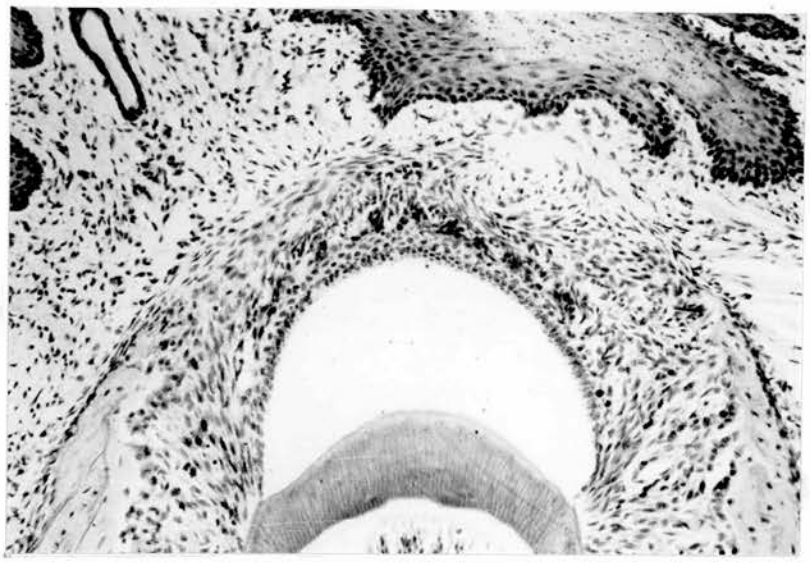

Fig. 12. 1 hour autoradiograph of an erupting 3rd molar tooth of a 25 day old mouse.

Stained haematoxylin. Orig. mag. X80.

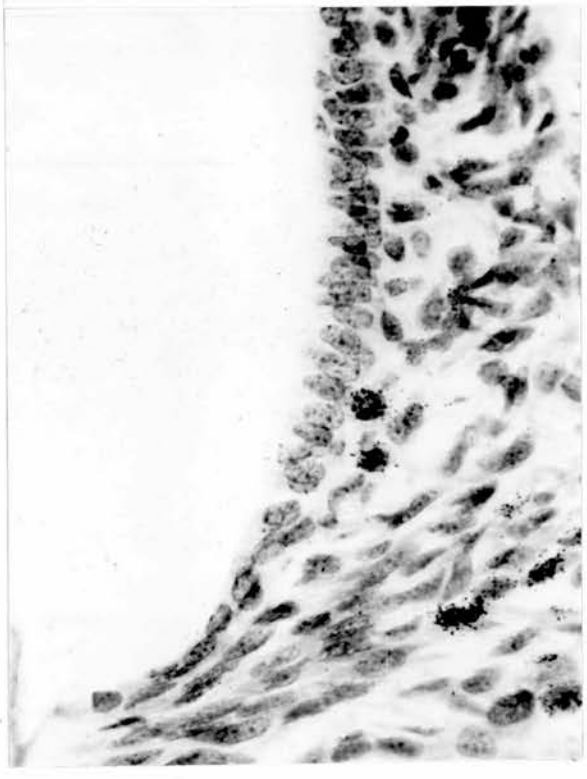

Fig. 12a. Higher power autoradiograph of the section in Fig. 12., showing labelled cells in the single cell layer stratum intermedium.

Stained haematoxylin. Orig. mag. X320.

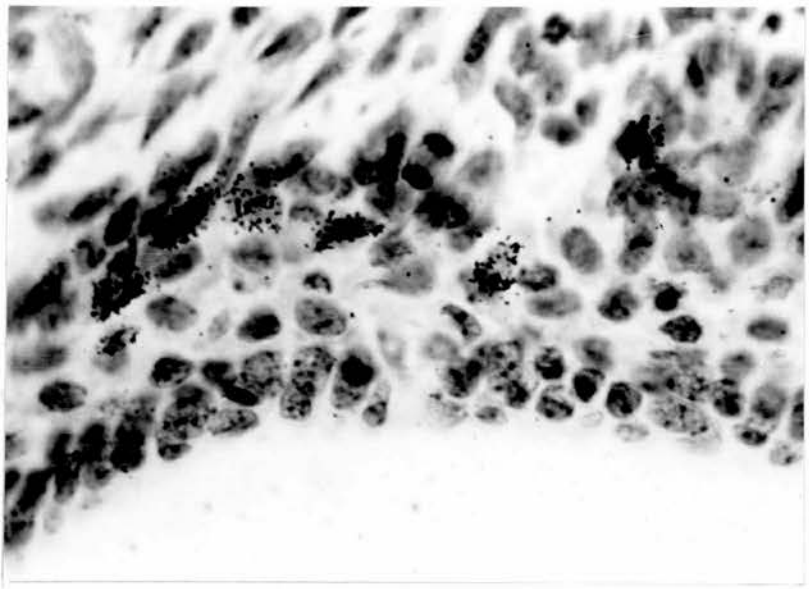

Fig. 12b. High power autoradiograph of the section shown in Fig. 12. In the proliferating epithelium over the crown, labelled cells are seen at different levels as well as an unlabelled mitosis in telophase.

Stained haematoxylin. Orig. mag. X320.

were still rounded, to the squamous cells adjacent to the down-growing oral epithelium. (Figs. 13 & 13a).

To the side of the crown a repeat of the findings described over the crown in Group I animals was observed, namely, isolated islets of epithelial cells with rounded nuclei and cytoplasm in continuity with the single cell layer of the stratum intermedium. 1 hour autoradiographs demonstrated that the cells in these islets were preparing for division.

In Group III animals the enamel epithelium over the 3rd molar crown had completely fused with the oral epithelium, and squamous epithelium was seen throughout. The ameloblasts had lost, or were losing their columnar form except in the fissures of the teeth and there was evidence of nuclear pyknosis and karyolysis. Autoradiographs at 1 hour were similar to those in the Group II animals, namely, labelled cells at various levels in the reduced enamel epithelium.

In all three animal groups 4 hour autoradiographs gave a similar picture to the 1 hour, whilst, by 12 and 24 hours, because of cell division, the number of labelled cells had increased but there was a corresponding decrease in the number of silver grains over each labelled nucleus.

2 and 3 day autoradiographs of the Group I animals showed the molar teeth in part eruption. (Fig. 14). At this period, the original labelled cells had divided several times as demonstrated by the very diluted labelling. Such an observation was most apparent in the epithelium adjacent to the cement enamel junction. (Fig. 14a). However, over the centre of the crown where the main

Fig. 13. 1 hour autoradiograph of the erupting 3rd molar tooth of a 26 day old mouse.

Stained haematoxylin. Orig. mag. X80.

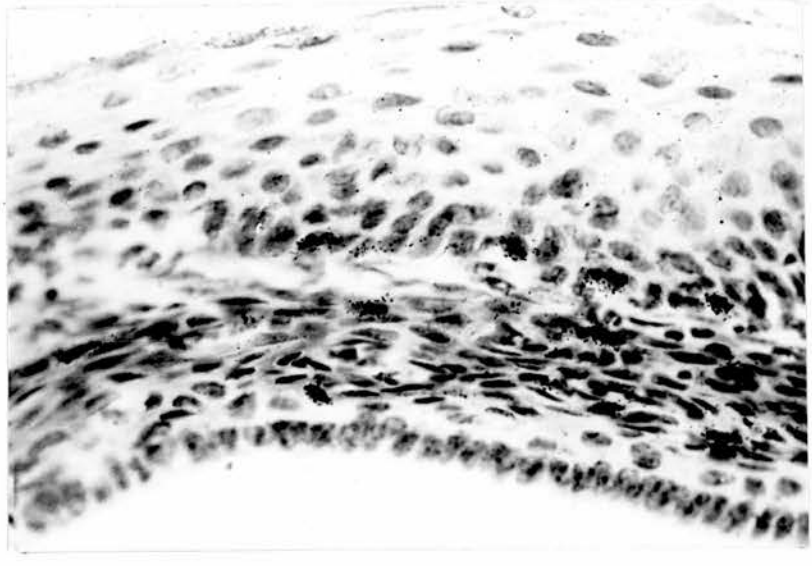

Fig. 13a. Higher power view of the autoradiograph in Fig. 13. Generalized labelling with H^3-thymidine is seen at all levels of the epithelium. Some labelling in cells adjacent to the unlabelled ameloblasts is shown.

Stained haematoxylin. Orig. mag. X320.

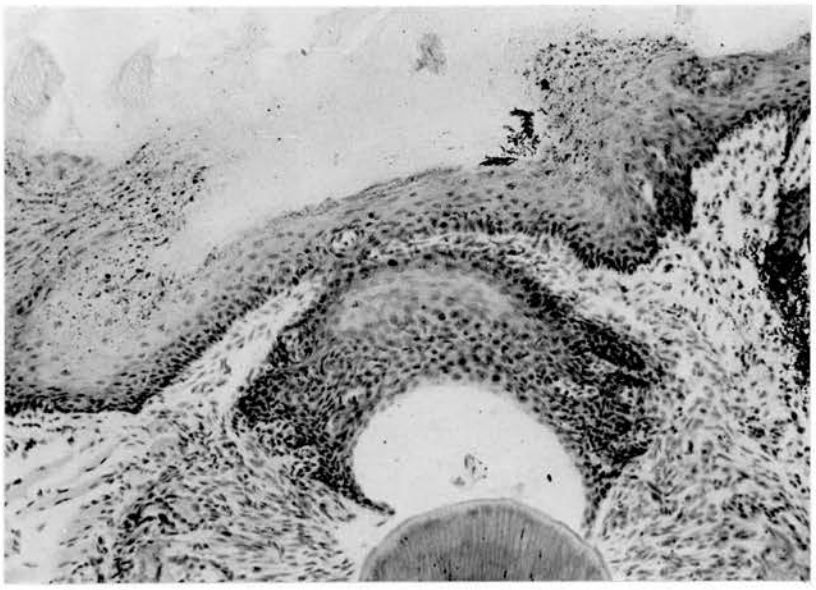

Fig. 14. Autoradiograph of an erupting 3rd molar tooth of a mouse, 3 days after injection of H^3-thymidine.

Stained haematoxylin. Orig. mag. X80.

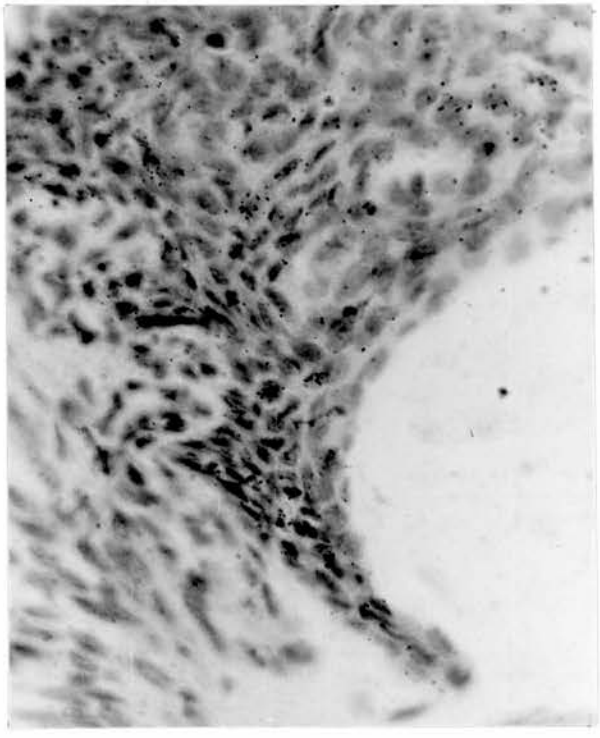

Fig. 14a. Autoradiograph from the same section as seen in Fig. 14., 3 days after injection of H^3-thymidine showing only dilute labelling in the reduced enamel epithelium next the cement enamel junction.

Stained haematoxylin. Orig. mag. X320.

histological observation was the presence of an area devoid of cells with a definite morphology, several heavily labelled cells were found. (Fig. 14b). Examination of these autoradiographs under polarized light demonstrated that this zone had positive birefringence similar to the keratinized layer of the oral epithelium. (Fig. 15). In Groups II and III, by the second and third day intervals after injection, the teeth had fully erupted. Half-labelled cells were still observed in the oral epithelium of the completed gingival papilla whilst diluted labelling was seen near the cement enamel junction. The keratin band observed in the Group I animals over the crown now extended apically towards the cement enamel junction. Occasional labelled cells were observed in the keratin which acted as a separating layer between the oral and the now squamous reduced enamel epithelium. (Fig. 16).

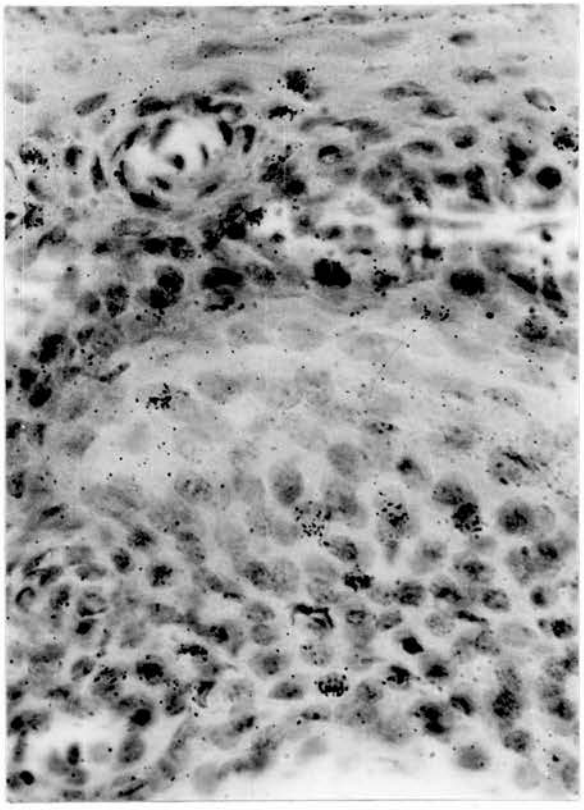

Fig. 14b. Higher power autoradiograph of the section shown in Fig. 14. Taken at the junction of the oral, downgrowth and reduced enamel epithelium, labelled cells are seen at various levels within the tissue. Between the reduced enamel and downgrowth epithelium is a zone of cells with poor staining properties.

Stained haematoxylin. Orig. mag. X320.

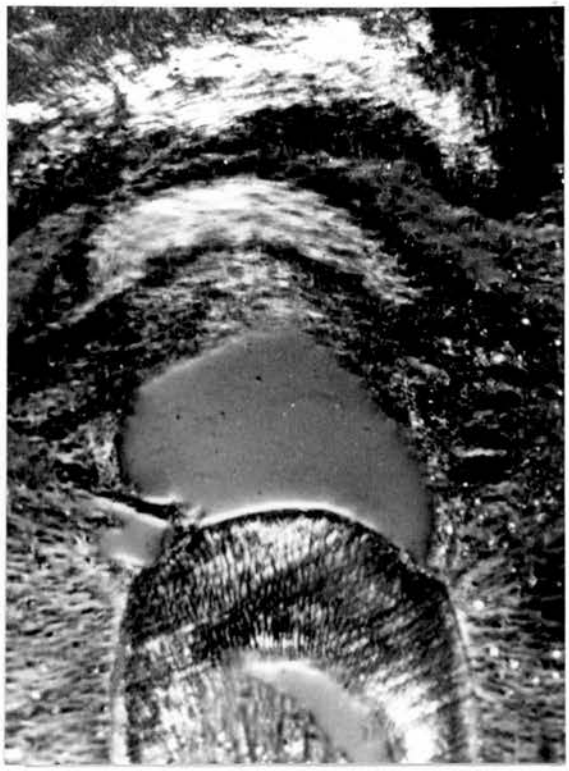

Fig. 15. Photomicrograph, in polarized light, of section illustrated in Fig. 14. Positive birefringence is shown in the surface keratinized layer of the oral epithelium and also at the junction between downgrowth, oral and reduced enamel epithelium.

Orig. mag. X40.

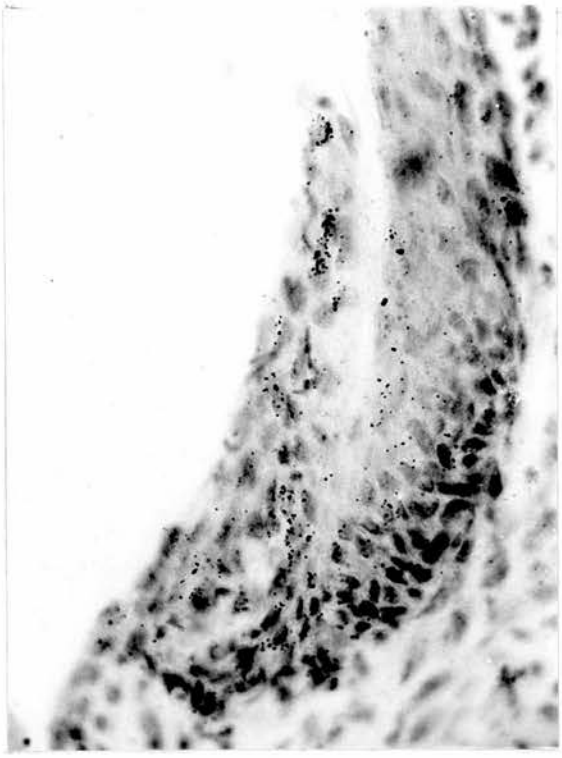

Fig. 16. Two days after injection of H^3-thymidine, this autoradiograph shows a section of the gingivae around a newly erupted 3rd molar tooth. Diluted labelling of cells is seen throughout the reduced enamel and downgrowth oral epithelium. A keratin band separates the two epithelia.

Stained haematoxylin. Orig. mag. X320.

OBSERVATIONS ON ORAL EPITHELIUM.

Both histological and autoradiographic changes were observed in the oral epithelium over the erupting tooth.

While the tooth was deep in the alveolar bone, the overlying oral epithelium had similar characteristics to the rest of the lining epithelium of the oral cavity. Rete pegs were present and at the 1 hour and 4 hour intervals, labelled cells were confined to the basallayer. (Figs. 17 & 17a).

As the tooth crown approached the oral epithelium, however, the degree of labelling at the 1 hour interval was first increased and then reduced. With this reduction there was an observed loss of rete peg formation. (Figs. 18 & 18a).

To record this autoradiographic observation, counts were made from 1 hour autoradiographs of 1,000 basal cells of the oral epithelium over the tooth crown before and during eruption. The initial count showed an average of 100-120 labelled cells per 1,000 basal cells. This increased to 200-250 at the start of tooth eruption and was reduced following the loss of rete peg formation to the original figure of 100 and less, as the reduced enamel epithelium coalesced with the oral epithelium. The increase in labelling was observed always in the epithelial prolongations on either side of the rete pegs.

At 12 and 24 hours the epithelium showed division of cells and "twin cell" labelling was then apparent. (Fig. 19). The "marked cells" were still found in the basal layer but there was a dilution of labelling corresponding to the cell mitosis. This was demonstrated by determining the grain count from 100 labelled

Fig. 17. Autoradiograph of an erupting 3rd molar tooth 1 hour after injection of H^3-thymidine. At this stage of eruption rete pegs are present in the oral epithelium over the tooth crown.

Stained haematoxylin. Orig. mag. X80.

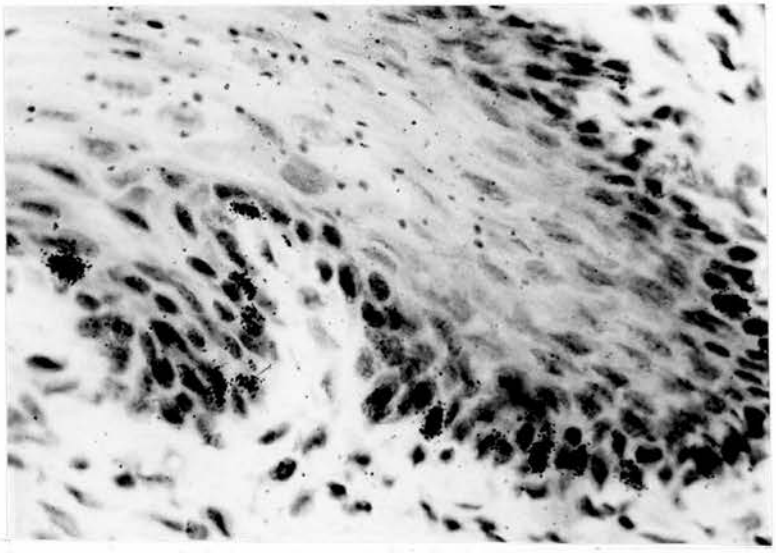

Fig. 17a. Higher power view of the autoradiograph in Fig.17. Large numbers of labelled cells are seen in the basal layer of the epithelium.

Stained haematoxylin. Orig. mag. X320.

Fig. 18. Autoradiograph 1 hour after injection of H^3-thymidine of a part erupted 3rd molar tooth. Rete pegs in the oral epithelium over the crown are now absent. cf. Fig. 17.

Stained haematoxylin. Orig. mag. X80.

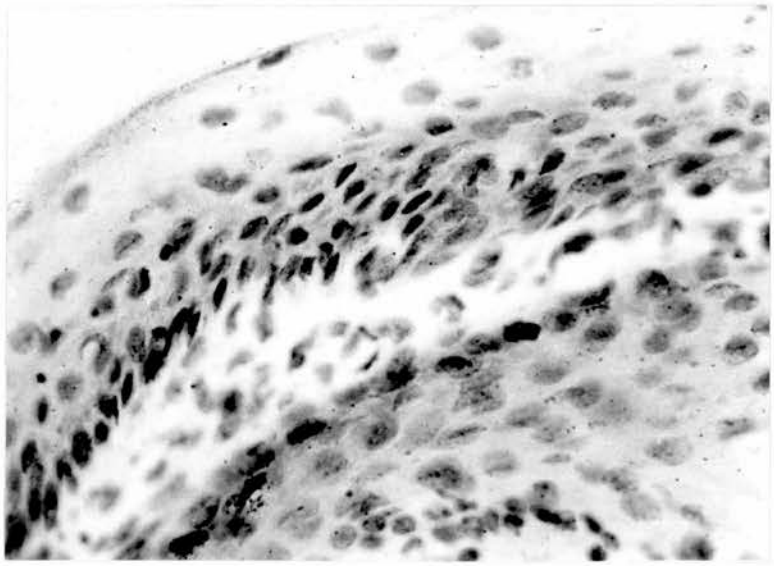

Fig. 18a. 1 hour autoradiograph of oral epithelium from section shown in Fig. 18. Note lack of labelled cells in the basal layer of the oral epithelium. cf. Fig. 17a.

Stained haematoxylin. Orig. mag. X320.

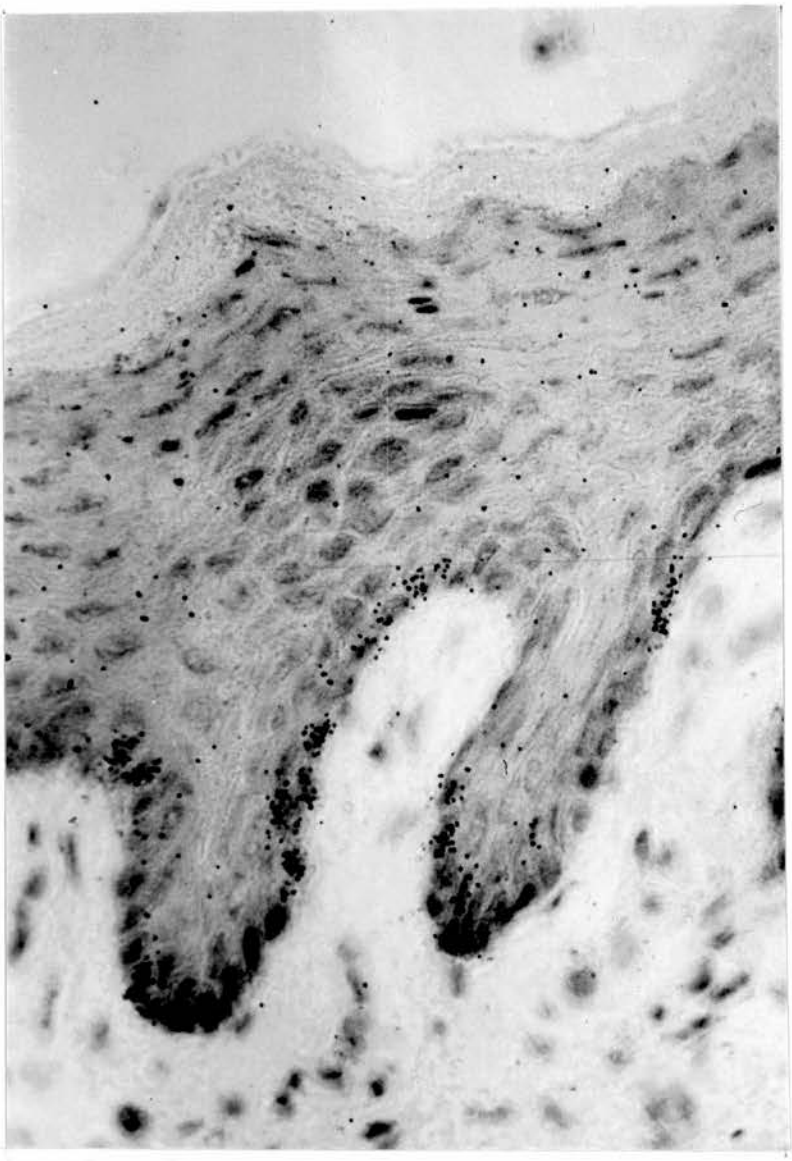

Fig. 19. An autoradiograph of oral epithelium 24 hours after injection of H^3-thymidine. "Twin cell" labelling, viz., cells with equal grain counts, are seen in the basal layer of epithelium.

Stained haematoxylin. Orig. mag. X320.

cells at both the 1 hour and 24 hour intervals. An average of 60 grains per cell was found at 1 hour compared with 25 grains at the 24 hour interval.

At the 2 and 3 day intervals the original labelled cells of the basal layer had divided more than once and the first division progeny were observed in the prickle cell layer. In areas where mitosis was active this observation was most apparent. (Fig. 20). Where, however, the erupting tooth had been in close proximity to the oral epithelium very few labelled cells were seen.

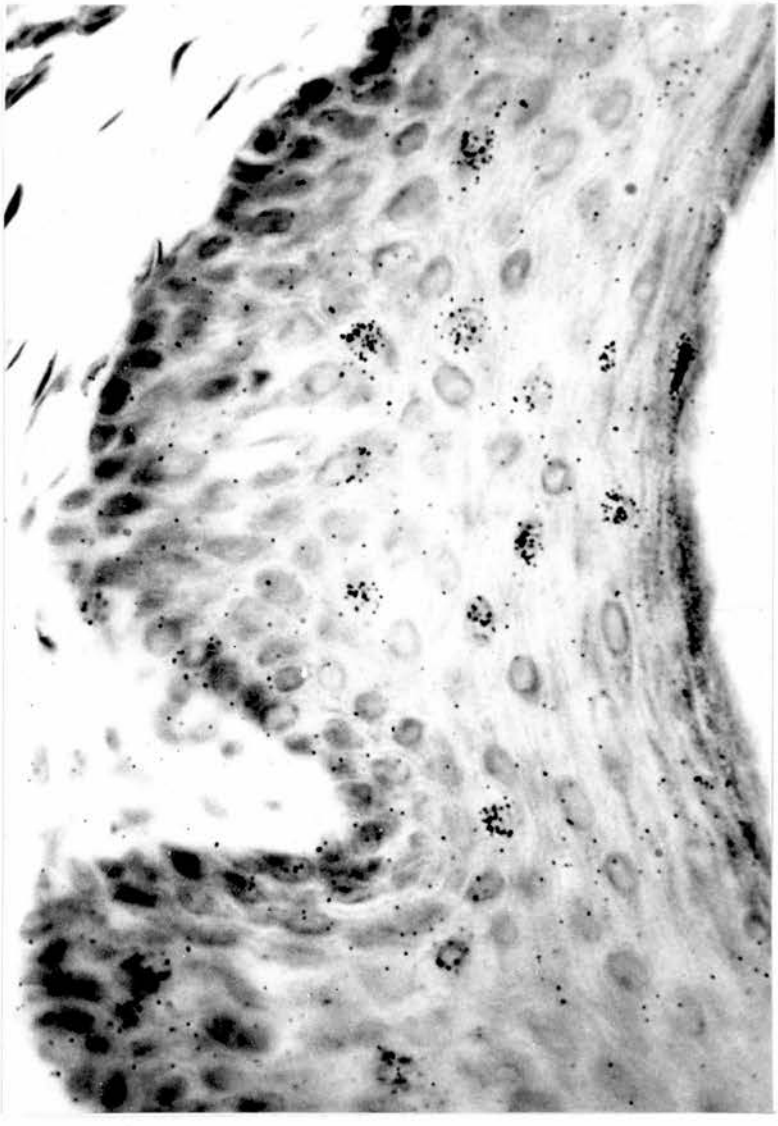

Fig. 20. 3 days after injection of H^3-thymidine, an autoradiograph of oral epithelium illustrating the migration of half-labelled cells into the prickle and superficial layers of the tissue.

Stained haematoxylin. Orig. mag. X320.

DISCUSSION.

The pattern of mitoses in the oral epithelium during the eruption process has been shown by autoradiography to vary according to the position of the tooth crown and the passive role of this tissue which has been described in the rat by Uohara (1959) cannot be supported. Observations forced the conclusion that the progenitor sites for the downgrowth of oral epithelial cells were in the epithelial prolongations adjacent to the rete pegs and that the proximity of the tooth crown in some way initiated this mitotic spurt. The cause of this stimulation is unexplained, but Bullough (1962) has shown that hormones of the glucocorticoid complex are most probably responsible for increased mitosis in a tissue.

In the latter stages of tooth eruption the overlying oral epithelium exhibited little or no mitosis and it would seem that Uohara (1959) drew his conclusions concerning the passive role of oral epithelium from observations at this period of gingival formation.

Considering the dental epithelium in the cervical loop of the enamel organ, premitotic labelling of cells at the 1 hour interval was concentrated in the pre-ameloblasts and stratum intermedium, with occasional labelling also in evidence in the outer enamel epithelium. Supporting these autoradiographic observations was the fact that, with the exception of the stellate reticulum, all of these cell populations presented histological evidence of mitotic figures. During later time intervals of 12, 24 and 48 hours, observations showed labelling in the stellate reticulum and a corresponding dilution of labelling in the stratum intermedium. It is suggested therefore that the cells of the stellate reticulum

are derived from the stratum intermedium by cell mitosis and migration. Such a fact is in agreement with the findings of Hunt & Paynter (1963) who showed a similar phenomenon in an autoradiographic study of the molar teeth of guinea pigs.

Following collapse of the stellate reticulum, no further labelling in the stratum intermedium was observed until the tooth crown was approaching the oral cavity. While the tooth was low in the alveolar bone, the stratum intermedium consisted of a single cell layer and preparation for mitosis was shown in cells adjacent to the shortened ameloblasts. As a phenomenon, this was first seen over the crown of the tooth but with eruption, similar observations were made laterally and towards the cement enamel junction. Meantime, division of the stratum intermedium cells, shown by dilution of labelling, caused an expansion of the reduced enamel epithelium. Morphologically these new cells were identical to the parent cell and had a rounded cytoplasm and nucleus.

The cellular expansion of the stratum intermedium seemed related to the position of the tooth with the alveolar bone crest. Beneath this position and extending to the cement enamel junction, the stratum intermedium was a single cell layer whilst above the crest it increased through the formation of cell islets to a layer 3-5 cells deep. When union with the downgrowing oral epithelium took place, labelled cells at the 1 hour interval were found at different levels of the fused epithelium. The labelled cells of the downgrowth epithelium were easily recognized since they were associated with a definite basal layer. In the enamel epithelium, however, they were found at different levels. The autoradiograph

in Fig. 13a, 1 hour after injection of H^3-thymidine, demonstrates this point. At this time interval, no cell movement can have occurred, yet the labelled cells are present throughout the whole thickness of the "mixed" epithelium over the erupting tooth crown.

At the 24 hour interval, divisions of these labelled cells had taken place, as demonstrated by "twin cell" formation and equivalent dilution of silver grains. The pressures from tooth eruption and cellular increase effected a change in cell morphology and flattened squamous cells were formed.

The presence of a keratin band in the epithelium over the tooth was interesting. Formed at the junction of the oral downgrowth epithelium and the reduced enamel epithelium, it persisted as a separating layer between these epithelia after tooth eruption. How it is first formed and from which epithelial tissue is open to argument. Oral epithelium of the mouse is keratinized on the surface and it can therefore form keratin. Chase (1929) and Hunt & Paynter (1959 : 1963) have contended that the reduced enamel epithelium too has the capacity to form keratin but there was no definite evidence of such a phenomenon in this present study. Some half-labelled cells were seen (Fig. 14b) in the centre of the keratin band but by their position they could have been derived from the basal cell layer of the downgrowth oral epithelium.

The exact mechanism initiating mitosis and expansion of the reduced enamel epithelium is not understood but, in this study, it appeared to be in some way related to the position of the tooth to the alveolar bone crest.

Baume (1952), Hunt & Paynter (1959 : 1963) have suggested that

proliferation of these epithelial cell populations only follows connective tissue destruction. However, there was little evidence of this in the present investigation. Labelling of fibroblasts was negligible in autoradiographs of this area and staining was ill-defined. The connective tissue was frequently composed of a series of fine radial whorls devoid of an organized stroma but this was not considered absolute evidence of tissue destruction.

The crest of the alveolar bone in the fully erupted tooth of the mouse was found directly opposite the cement enamel junction. At this period the connective tissue fibres of the periodontal membrane appeared to be finally orientated and little evidence was now seen of DNA synthesis within them. This was in direct contrast to the autoradiographic observation on the actively erupting tooth. Alldritt (1961) discussed the controlling influence of these organizing collagen fibres uniting the tooth to alveolar bone crest and considered that the length and morphology of the mature gingivae was decided by them. The lack of autoradiographic evidence of mitosis when the mature fibres had been formed would give support to Alldritt's hypothesis.

In all three experimental groups of animals it was only possible to recognize the ameloblast and stratum intermedium cell layers of the original enamel organ 2-3 days before eruption of the 3rd molar teeth. Depending upon the stage of tooth eruption, the stratum intermedium consisted of a layer 1 - 5 cells deep and cells of this layer were undergoing DNA synthesis prior to mitosis. It would seem therefore from this study that the cells of the stratum intermedium have a potential for division

and proliferation. This observation is in agreement with the findings of Johnson & Bevelander (1957), Hunt (1959), Uohara (1959) and Hunt & Paynter (1963). The opinion of these workers was that the cells of the stratum intermedium were alone responsible for the formation of the layer of cells of reduced enamel epithelium which, with oral epithelium, formed the gingivae.

McHugh (1959) did not find evidence of any cell division in the stratum intermedium. Instead, he suggested that the stratum intermedium was in degeneration, using, as an argument, the finding of Symons (1955) who demonstrated a high alkaline phosphatase activity in this cell layer during matrix formation of enamel, which was lost after the matrix had been completed. No evidence of degeneration was, however, found in this present investigation within the stratum intermedium. It still had its individual cell outline two days before eruption and preparation for cell division was found within the layer.

On completion of enamel matrix formation, Williams (1896), Wassermann (1944) and McHugh (1959) were all of the opinion that the stratum intermedium disappeared and that outside the ameloblasts was found an amorphous layer of cells which McHugh (1959) considered to be a combination of outer enamel epithelium and stellate reticulum. Of all the cells of the enamel organ, other than the ameloblasts, only the stratum intermedium cells remained unquestionably recognizable after collapse of the stellate reticulum. But can they be retained without some purpose? Ten Cate (1961) has shown that prior to the onset of calcification the stratum intermedium cells can replace ameloblasts. The present hypothesis is that in addition they proliferate to form buds of epithelium which

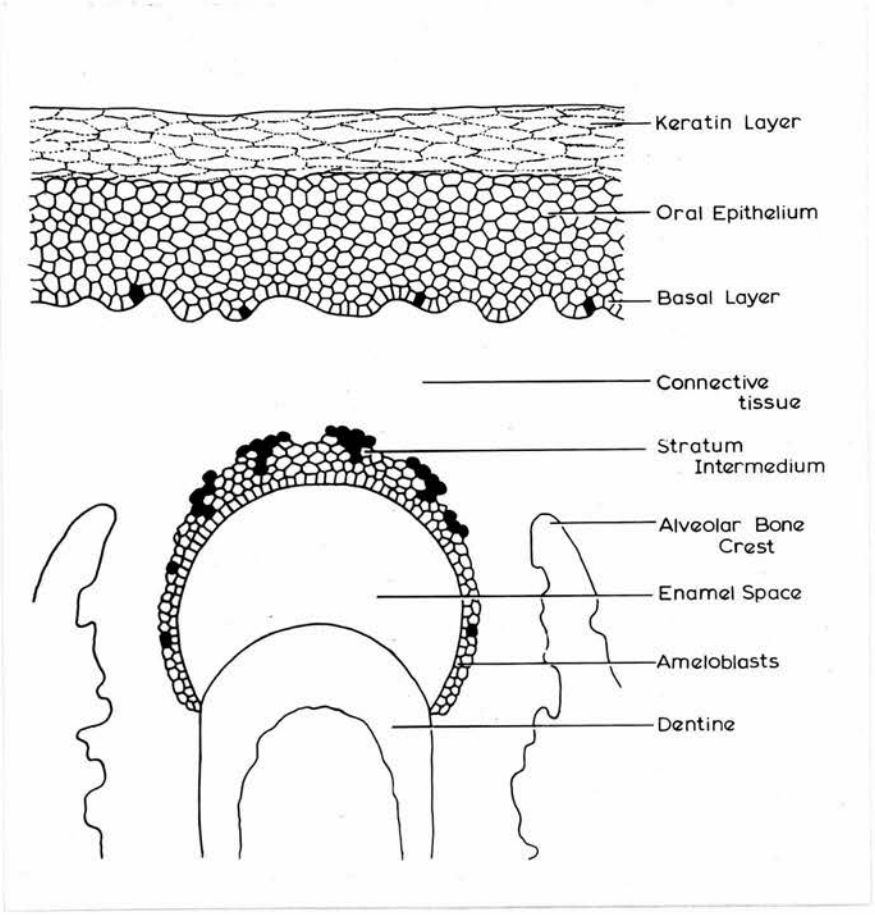

Fig. 21. A diagrammatic illustration of the pattern of mitoses in epithelium over an erupting mouse molar tooth. Black circles indicate the position of labelled cells as seen by autoradiographs at this stage of eruption 1 hour after injection of H^3-thymidine.

Beneath the alveolar crest, labelling is present in the single cell stratum intermedium. Above the crest, proliferation of this layer has caused expansion of the reduced enamel epithelium.

coalesce with cells of the overlying oral epithelium to form the squamous cells of the mature gingivae.

In this experiment the complete epithelial mitotic pattern was difficult to follow even when using animals of different age groups. The observations made, however, prompt the suggestion that the immediate changes in the epithelium over the erupting mouse molar tooth can be described in four stages. These are best illustrated by considering the autoradiographic picture ONE HOUR after injection of H^3-thymidine, i.e., before labelled cell migration can occur :-

Stage 1. This is characterized by proliferation of the cells of the stratum intermedium adjacent to the ameloblasts and is seen after the enamel maturation process is complete at a time when the ameloblasts are shortened, the stratum intermedium reduced to a single cell layer and while the tooth crown is below the alveolar bone crest. Following on this there is proliferation of the cells of the stratum intermedium to form a layer 2-5 cells deep. This proliferation forms irregular cell groups devoid of an organized basal layer. External to these, the basal cells of the oral epithelium show an increase in mitosis. Initially occurring over the crown of the tooth, this whole stage is later seen to extend apically towards the cement enamel junction. (Fig.21).

Stage 2. In this, coalescence and organization of the individual cell groups occurs to form an outer basal cell layer in conjunction with the oral epithelium. At this stage cell mitoses are found at various levels of the unified epithelium - some derived from the enamel epithelium and some from the oral epithelium. The downgrowth of oral

epithelium, however, shows the labelled cells in the basal layer only. (Fig. 22).

Stage 3. The continued proliferation and movement of cells towards each other from the separate progenitor sites causes thickening of the epithelium and the formation of a keratin layer which persists even after eruption to separate the two epithelia. (Fig. 23).

Stage 4. With eruption, the epithelial area having the highest mitotic activity is seen in the region of the cement enamel junction in a position just above the alveolar bone crest. The shape of the mature gingival papilla of the mouse molar is now determined. (Fig. 24).

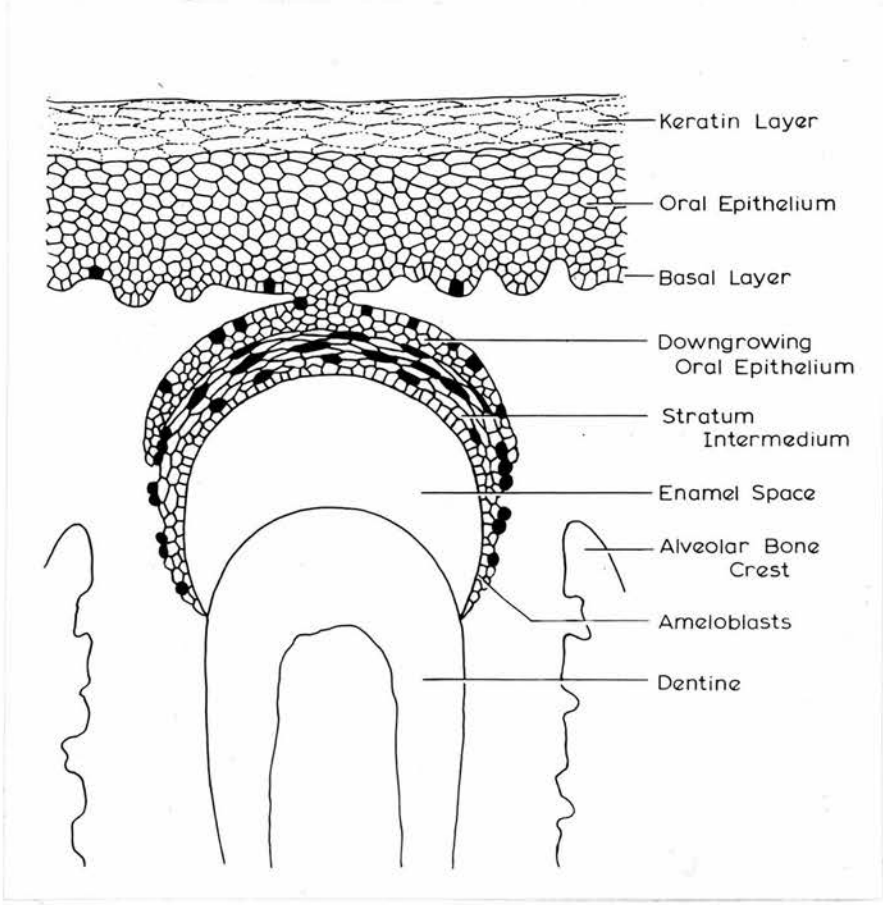

Fig. 22. An autoradiographic diagram in which black circles indicate the pattern of cell mitoses in the epithelium over an erupting mouse molar tooth 1 hour after injection of H^3-thymidine. By this stage, coalescence of epithelium has occurred. cf. Fig.21.

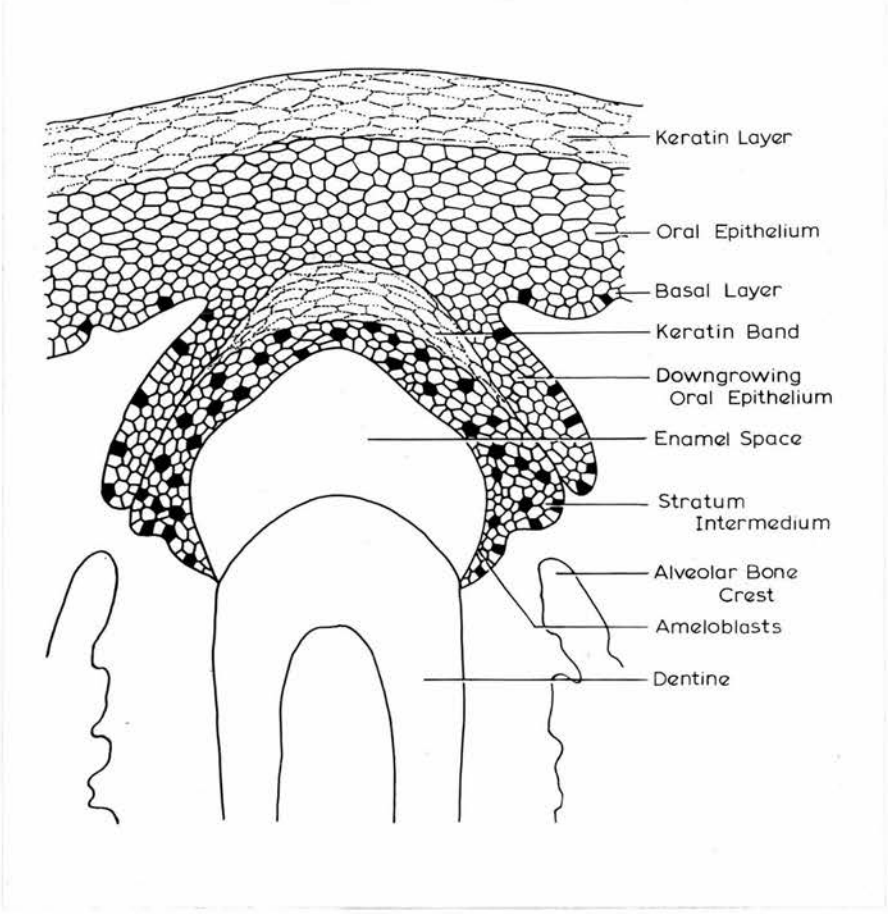

Fig. 23. Black circles, representing undivided labelled cells of an autoradiograph, illustrate the distribution of mitoses in epithelium over an erupting mouse molar tooth. Coalescence of the epithelia has occurred and keratin separates the oral and proliferated stratum intermedium.

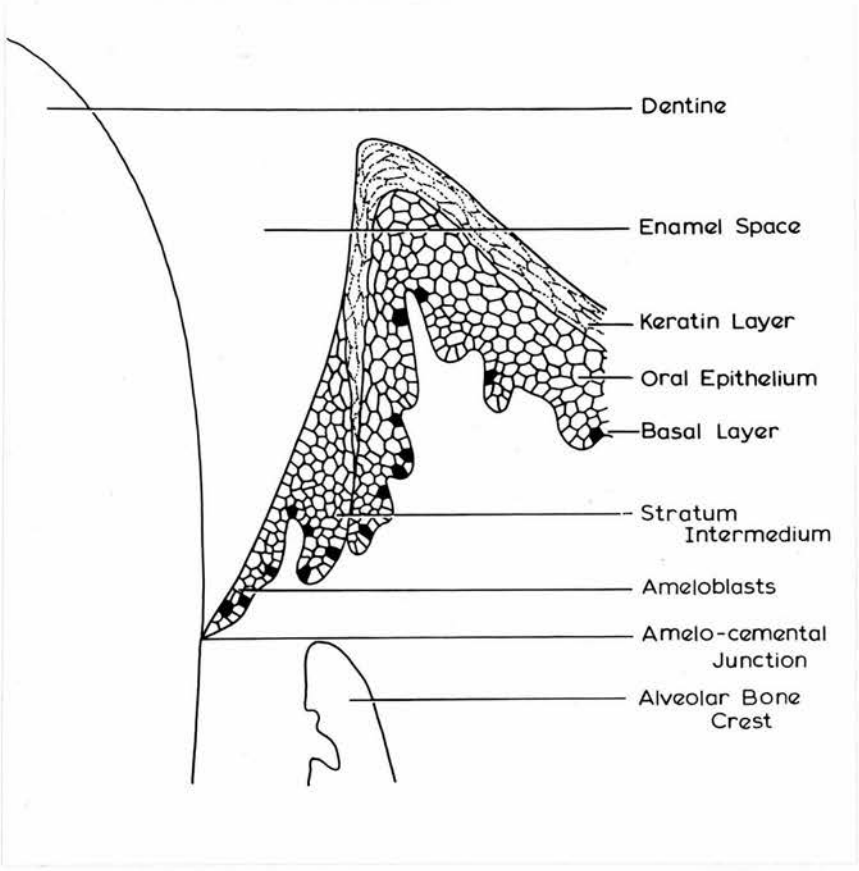

Fig. 24. 1 hour autoradiographic diagram of the retro-molar papilla of a mouse, immediately after tooth eruption. An extension of keratin separates the epithelia. The mitotic pattern of cells is indicated by the black circles.

CONCLUSIONS.

1. In considering the histogenesis of gingival epithelium of the mouse molar teeth, two proliferative sites were recognized - oral epithelium and reduced enamel epithelium.

2. In the case of oral epithelium, cell mitosis and proliferation occurred from the basal epithelium of the oral tissue overlying the tooth crown.

3. In enamel epithelium the only cell layer of the enamel organ showing proliferative capabilities, as recognized by H^3-thymidine autoradiography, was the stratum intermedium and it was therefore regarded as the essential component in the histogenesis of this gingival structure.

4. The coalescence of the oral and proliferating stratum intermedium was marked by a separating band of keratin which persisted after the tooth erupted.

5. DNA synthesis in the connective tissue forming the mature collagen fibres of the periodontal membrane dramatically decreased with the positioning of the cement enamel junction opposite the alveolar bone crest. When this position was reached, the gingival papilla had taken on its adult morphology.

S E C T I O N

III.

SECTION III.

THE GINGIVAE AROUND THE ERUPTED TOOTH

When a tooth has erupted into the oral cavity the gingival tissues undergo a process of maturation and become adapted to their normal functional adult size. The epithelial changes associated with tooth eruption have been described in Section II. In this the oral epithelium coalesced with the enamel epithelium and formed a stratified squamous epithelium throughout.

Over the years, a subject of great dispute has been the relationship between oral epithelium, enamel epithelium and the enamel surface of the tooth.

REVIEW OF LITERATURE.

In 1839, Nasmyth described a thin membranous acellular structure on the surface of the enamel which was afterwards referred to as Nasmyth's Membrane. Paul, in 1895, reported further on this tissue and described it as being composed of two layers, an outer layer of flattened epithelial cells and an inner thin hornified layer. Black (1915), after examination of demineralised tissue sections of tooth and periodontal tissues, concluded that a potential space existed between the surface epithelial cells of the gum and the enamel of the tooth. This concept was an accepted one until Gottlieb (1921) introduced the idea of "epithelansatz" or "epithelial attachment" in which he described again from histological sections an organic union of the gingival epithelium to Nasmyth's Membrane. It was claimed that, with eruption of the tooth, the superficial layers of the gingival epithelium keratinized

and were united with Nasmyth's Membrane. This binding layer of keratinized cells Gottlieb referred to as the "secondary enamel cuticle" to distinguish it from Nasmyth's Membrane which was then referred to as the "primary enamel cuticle". The supporters of such a theory of attachment of epithelium to enamel through an organic union were many and included Orban & Köhler (1924) : Orban & Mueller (1929) : Kronfeld (1930) : Bodecker & Applebaum (1934) : Manley (1936) : Toller (1939 : 1940) : Baume (1952 : 1953) : Butcher (1953) : Macapanpan (1954) : Ussing (1955) : Orban et al (1956) : Weinreb (1960) and Cohen (1962).

With the exception of Bodecker & Applebaum (1934), Toller (1939 : 1940) and Weinreb (1960), the attachment protagonists formed their opinions from observations on fully decalcified sections and were thus provided with a static rather than a dynamic concept of cell activity. In 1952, Waerhaug, in his monograph, "The Gingival Pocket", produced evidence of a more acceptable kind. In this, histological evidence, although still utilized, was supported by experimental material to demonstrate the attachment strength of the organic union with enamel. In one experiment thin stainless steel metal strips of 0.05 m.m. thick were introduced with a force of 5 grams into the gingival crevice of dogs and children. Radiographs showed that the strips were halted at the amelo-cemental junction of the teeth. Furthermore, post-experimental histology of the area failed to show any disruption of the epithelial cells at the surface of the lining epithelium. McHugh (1959) repeated these experiments on monkeys and his findings were in agreement with those of Waerhaug. Waerhaug (1952) also demon-

strated the rapid healing capacity of this gingival epithelium in dogs by introducing, between the tooth and the gingival epithelium, virulent bacteria and India ink as foreign bodies. Sacrificing the animals so treated in both of these experiments, it was shown that complete recovery of the gingivae could be expected even when gross ulceration of the pocket epithelium had been produced. In another experiment, Waerhaug (1953) showed how an epithelial lining with normal histological characteristics could be formed round acrylic root tips implanted into dog tooth sockets and Zander (1956) established such a picture against a cellulose acetate strip. Waerhaug (1956a) then demonstrated the reformation of the secondary cuticle and new epithelial lining against the enamel surface of the gingival pocket after its destruction with a diamond stone. He proposed the term "epithelial cuff" instead of "epithelial attachment", suggesting that instead of an attachment, there existed a close adaptation and adhesion of the epithelium to the enamel. Furthermore, that the epithelium itself was held in close apposition to the enamel by blood pressure and by the connective tissue fibres within the gingivae. (Waerhaug 1959). Orban et al (1956) in repeating Waerhaug's strip experiments, considered the attachment still to be an entity in normal gingival physiology. Baume (1952 : 1953) had a new concept to explain the attachment, namely, that it existed by tonofibrils or cellular bridges between the surface cells of the epithelium and the enamel surface. Waerhaug (1956b) however, was able to produce these tonofibrils against gold foil of replanted teeth in a monkey. McHugh (1959) in complete agreement with the Waerhaug concept suggested that no organic union could

possibly exist between the epithelium and the surface of the enamel since the cells on the surface of the gingival lining epithelium were degenerate. It would appear that the supporters of the attachment theory considered that the cells which formed the epithelium of the gingivae differed in their life cycles from cells in the rest of the oral epithelium. Baume (1953) was of the opinion that the epithelial attachment stability and firmness depended upon the longevity of the attached epithelium and the preservation of the vitality of the surface cells and attaching tonofibrils. Gottlieb (1927) believed that attachment only became severed when the enamel surface was non-vital. This was his explanation of "passive eruption", a term which he introduced to explain the movement of a tooth through the gingival epithelium or the gingival epithelium losing its contact from the tooth during life. Orban (1944) explained that this phenomenon of passive eruption occurred by a process of degeneration at the base of the gingival sulcus through bacterial and mechanical irritation. Becks (1929) suggested that passive eruption of a tooth occurred in association with a continual downgrowth of oral epithelium to unite with the epithelial attachment. Growth of the oral epithelium being a continuous process on the connective tissue side of the attachment, he further postulated that with increased age the gingival epithelium eventually extended to the cement enamel junction and in this way enclosed the original attached epithelium. Skillen (1930a) upheld Beck's theory by observations on rat material and Scott & Alldritt (1957) were of the same opinion as far as the rodent was concerned. The supporters of the static eruption con-

cept are thus at variance with the dynamic epithelial cuff adherents. In the one hypothesis, the epithelium against the tooth surface remained unchanged whereas, in the other it was considered to have the same biological principles of cell life and death as other epithelial tissues in the body.

It has already been established that growth and replacement of a tissue occurs by cell division. If the epithelium of the gingivae has a static cell population such as the corneal epithelium of the eye (Messier & Leblond 1960) then the supporters of the attachment theory could be correct, viz., that there is a static cell population in this area undergoing no change, as a result of which phenomenon a permanent union could be present between the gingival papilla and the enamel surface. If, however, there is evidence of mitosis in the tissue and the cells of the tissues are replaced from the basal cell layer and desquamated towards the enamel surface, then no form of permanent attachment to the enamel could occur; which would seem to be further evidence in support of the Waerhaug (1952) dynamic concept of gingival pocket epithelium.

The presence of mitotic figures in gingival epithelium has been described by Bass (1946): Hirt, Hartl & Mühlemann (1955) : Mühlemann & Hartl (1955) : Meyer et al (1956) : McHugh (1958 : 1959) : Greulich et al (1961) : Beagrie & Skougaard (1962) : Skougaard & Beagrie (1962) and Trott & Gorenstein (1963).

As discussed in Section I, cell populations of a tissue were typed according to their mitotic activity. Hair follicles, skin and mucous membranes had a renewing cell population in which the number of cells formed by mitosis equalled the number lost by desquamation (Wimber 1963). Another way of expressing a cell

population state was outlined by Messier & Leblond (1960). Depending upon the percentage of cells labelled with H^3-thymidine they defined three cell populations in an organism as follows :-

1. Static, in which no labelled nuclei are shown after injection, e.g., neurons of cerebrum, cerebellum and spinal cord.
2. Expanding cell population, where labelled nuclei are found at a level of 0.4% - 1% and seem to be present indefinitely; e.g., muscles and connective tissues.
3. Renewing cell population, where 3% or more labelled nuclei are present. This percentage increased after 24 hours and decreased 3-6 days later. It was found in skin and mucous membranes.

TRITIATED THYMIDINE AUTORADIOGRAPHY OF THE MATURE GINGIVAL TISSUE.

Is growth and cell replacement the same in the crevicular epithelium as in the remaining parts of the gingival papilla? Sections through mature mouse molar gingival tissue give an appearance which is peculiar to the rodent. Hirt, Hartl & Mühlemann (1955) described such sections in rats according to the diagram illustrated in Fig. 25. The development of this rodent gingival outline was discussed in Section II. In this, a keratin band developed with tooth eruption into a separating layer between the epithelial attachment and the downgrowing oral epithelium. Such a division is useful for the purpose of investigating any variation in cell life in the parts of the epithelium which constitute the rodent gingival papilla and using this arbitrary division of the mouse gingival epithelium along with H^3-thymidine autoradiography, it was decided to :-

(i) make a comparison of the labelling indices;

(ii) determine the type of cell population and renewal times of the three epithelial areas;

(iii) ascertain the progenitor sites for new cells;

(iv) suggest the migration pattern of cells after mitosis; and lastly,

(v) by application of the information gathered from these observations, to suggest the attachment relationship of epithelium to the tooth enamel surface.

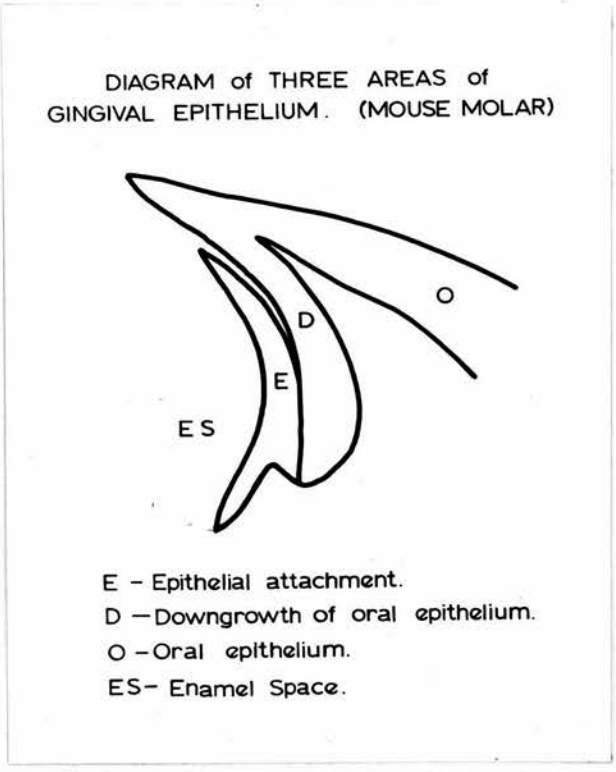

Fig. 25. Diagram of a section of the retromolar papilla of a mouse molar tooth indicating the three parts of epithelium making up this tissue.

(After Hirt, Hartl & Mühlemann (1955)).

PRESENT INVESTIGATION

The material for this study was made up of 15 male (Q strain) mice from the Animal Genetics Department of Edinburgh University. Certain strains of mice are prone to gingival disease (Baer & Lieberman 1960). Prior examination of the periodontal tissues of this strain, however, failed to show disease until the animals were 4-5 months old. Thus, for this investigation, they were selected aged 8 weeks $\pm$ 1 day. They had an average weight of 28 grams. It was decided to make the autoradiographic observations on the gingival tissues which make up the retro-molar papillae of these animals and since Mahn (1890) has shown that the last molar teeth of the mouse erupt at 28 days, the observations of this experiment were related to adult animals with fully erupted teeth.

Each mouse was injected subcutaneously with tritiated thymidine (specific activity 3 curies/milli-mole) at a dosage of 1 microcurie per gram body weight. Following injection, three animals were sacrificed 1 hour later and one animal at the intervals of 3 hours, 6 hours, 12 hours, 24 hours, 36 hours, 2 days, 3 days, 5 days, 7 days, 10 days, 12 days and 15 days.

At sacrifice, the vascular systems of the animals were perfused with 10% neutral formalin via the right atrium of the heart and following this the heads were removed, decalcified in a 10% solution of formic acid and double embedded in celloidin and paraffin wax. Sections were cut mesio-distally through the molar teeth at 6-8 microns and covered with autoradiographic stripping film (Kodak AR.10) as described by Pelc (1947). The prepared sections were stored in the dark at a temperature of 5°C for 21 days,

after which the exposed films were developed and fixed and the sections stained with dilute haematoxylin.

RESULTS*

From each animal 10 autoradiographs were selected at random and observations made from the retro-molar gingival papillae throughout the experiment. Counts of at least 1,000 cells were carried out under oil immersion at a magnification of 640 diameters for each area of epithelium.

1 hour interval.

At this interval, there was generalized labelling in all of the epithelium examined. (Fig. 26). The percentage distribution of labelled cells was 3.6% oral, 5.6% downgrowth and 6.7% in the attachment epithelium. (Table V). In the oral and downgrowth epithelium, labelled cells were confined to the basal layer, whereas, in the region of the epithelial attachment, some marked cells were in evidence on the surface of the layer in direct contact with the enamel surface. This was especially so in the tissues closely related to the cement enamel junction. (Fig. 27). A count of 250 labelled cells at this interval showed that each cell had an average of 65 silver grains over the nucleus.

3, 6 and 12 hour intervals.

At these intervals, a small increase in the labelling percentage was evident. The position of labelled cells was, however, the same as at the 1 hour period for all epithelial tissues.

* Some of the initial results of this experiment have been reported (Beagrie, G.S. & Skougaard, M.R. (1962) : Acta odont. scand. 20 : 15).

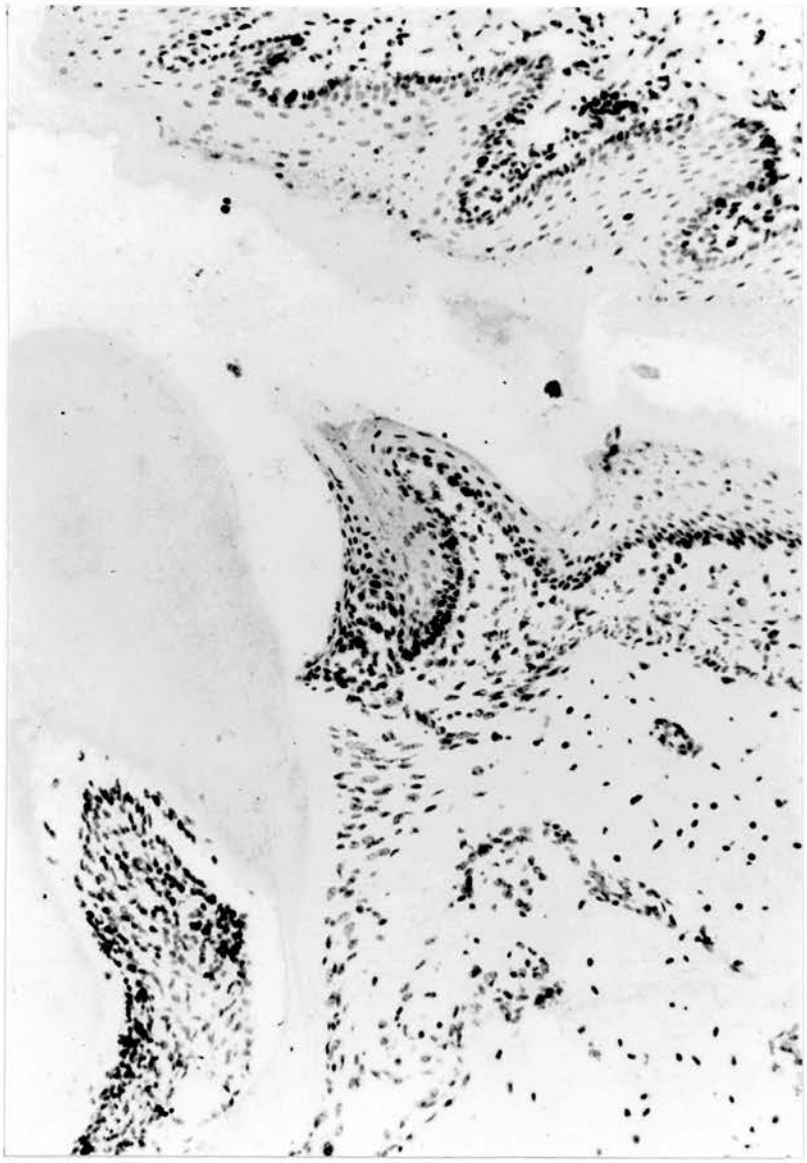

Fig. 26. Autoradiograph of the retro-molar papilla and cheek epithelium of a mouse 1 hour after injection of H^3-thymidine. Labelled cells are seen in the basal layers of all three epithelia.

Stained haematoxylin. Orig. mag. X80.

Fig. 27. Autoradiograph of the epithelial attachment 1 hour after labelling with H^3-thymidine. The majority of labelled cells are confined to the basal layer next the cement enamel junction. One labelled cell is shown on the surface of the epithelium adjacent to the enamel space.

Stained haematoxylin. Orig. mag. X320.

24 hour interval.

A notable increase in labelled cells was now demonstrable and with this increase the cells exhibited a corresponding dilution in the numbers of silver grains over their nuclei. A count of 250 labelled cells now gave an average grain count of 22 grains compared with the reported 65 grains at the 1 hour interval. These were obviously daughter cells of the original parent cell. For oral and downgrowth areas the "twin" cells were found still in the basal layer. (Fig. 28). In the attachment epithelium, fewer labelled cells were observed at the cement enamel junction. Labelling indices for this 24 hour interval were - oral epithelium 7.25%, downgrowth epithelium 10.50% and attachment epithelium 14.75%. (Table V).

At 36 hours and 48 hours after injection, half-labelled cells were observed throughout the more coronal parts of the epithelial attachment. Diluted labelling was in evidence near the cement enamel junction. In oral and downgrowth areas the labelling was 1-3 cells deep into the prickle cell layer. (Fig. 29).

In the attachment area at the 3-5 day interval only diluted cell labelling was present near the cement enamel junction although some half-labelled cells were present at the coronal tip of the epithelium. (Figs. 30 & 31). This was in contrast to the oral epithelium (Fig. 20) where cell labelling was generalized throughout the complete epithelial cell layers. The labelling indices at 3 days were respectively, 3.2%, 1.8%, 2.6% and at 5 days 2.1%, 1.2% and 0.4% for oral, downgrowth and epithelial attachment areas. (Table V).

Fig. 28. Autoradiograph of the retro-molar papilla of a mouse 24 hours after injection of H^3-thymidine. "Twin cell" labelling is evident in all parts of the papilla.

Stained haematoxylin. Orig. mag. X320.

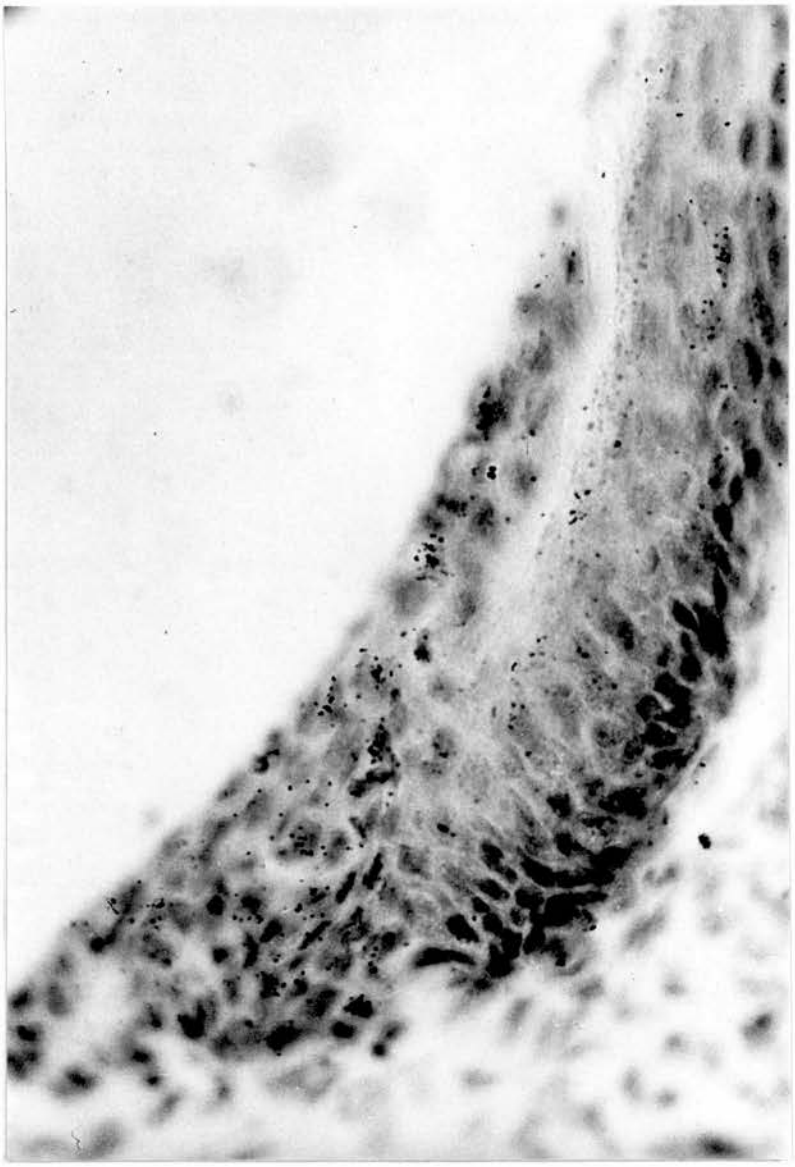

Fig. 29. Autoradiograph of a mouse retro-molar papilla 48 hours after injection of H^3-thymidine. Half-labelled cells have migrated to the surface of the epithelial attachment next the enamel space.

In downgrowth epithelium, half-labelled cells are seen a few cells in from the basal layer.

Stained haematoxylin. Orig. mag. X320.

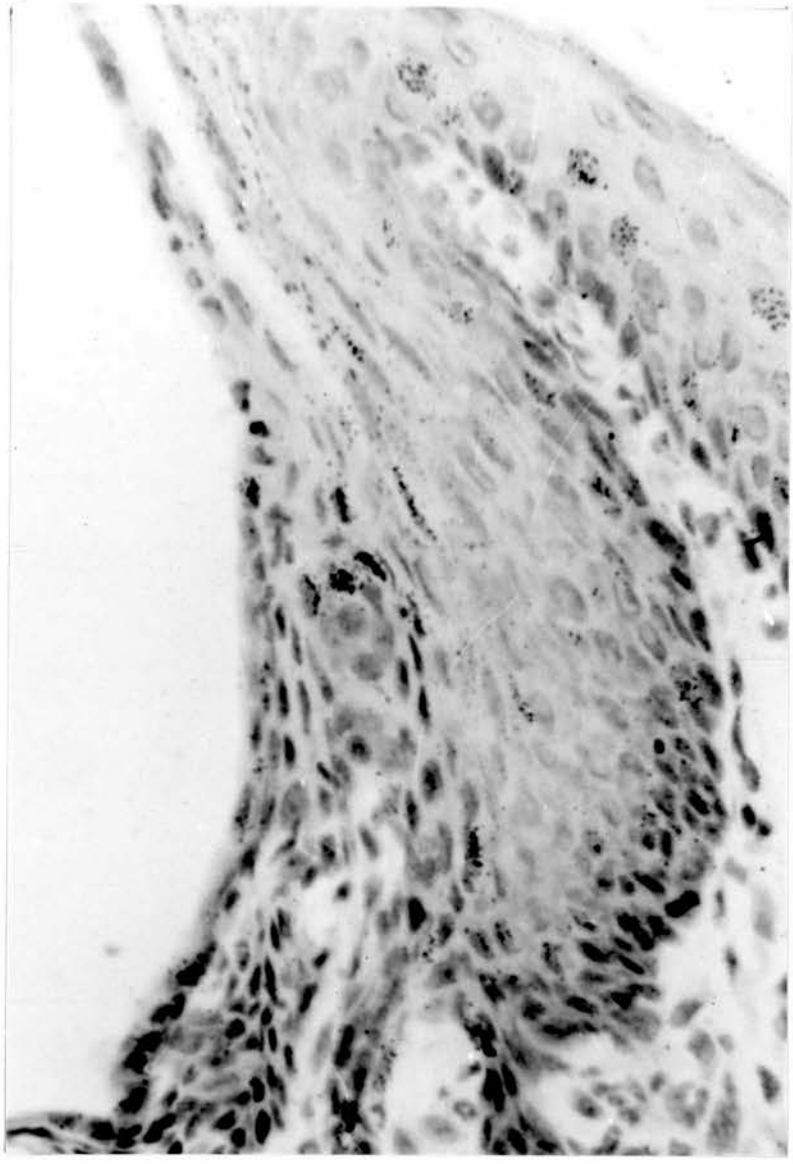

Fig. 30. 3 day autoradiograph of a mouse retro-molar papilla, showing migration of half-labelled cells into the spinous layer of the oral and downgrowth epithelium. In the epithelial attachment dilute labelling is present next the cement enamel junction while half-labelled cells are seen in the coronal tip.

Stained haematoxylin. Orig. mag. X320.

Fig. 31. Autoradiograph of the retro-molar papilla of a mouse 5 days after injection of H^3-thymidine. Two half-labelled cells are being desquamated at the coronal tip of the epithelial attachment towards the enamel space.

Stained haematoxylin. Orig. mag. X320.

TABLE V

Radioactive Index (% labelled cells) at different time intervals after injection of H^3-thymidine

	1 hour	24 hours	3 days	5 days
Epithelial Attachment	6.7	14.75	2.6	0.4
Downgrowth Oral Epithelium	5.6	10.5	1.8	1.2
Oral Epithelium	3.6	7.25	3.2	2.1

(count of 1,000 cells each area)

By 10 and 12 days after injection, half-labelled cells were lost, even in oral and downgrowth epithelium and only connective tissue showed evidence of DNA synthesis. (Fig. 32).

TYPE OF CELL POPULATION.

It has previously been shown that the type of cell population in a tissue is dependent upon the labelling indices at particular time intervals.

At the 1 hour interval after injection of H^3-thymidine, all of the epithelial cell populations comprising the retromolar papillae in the present investigation had a labelling index of over 3%. Furthermore, at the 24 hour interval, the indices had all increased to a figure greater than the initial 1 hour index and by the end of 3 days had again fallen to a figure below those recorded at the 24 hour period. (Table V). Such data fulfil the criteria of Messier & Leblond (1960) for a tissue with a renewing cell population. Thus all epithelia in this examination can be so designated.

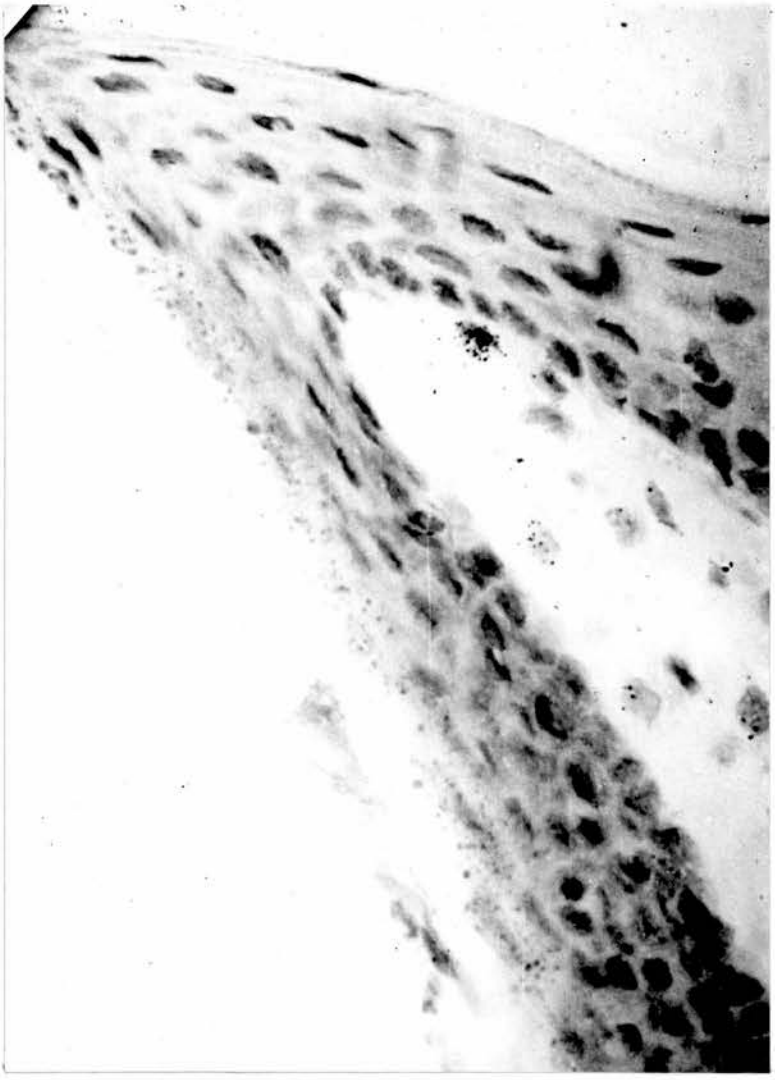

Fig. 32. Autoradiograph of the mouse retromolar papilla 12 days after injection of H^3-thymidine. Only very dilute labelling of cells (1-2 silver grains) can be seen in the epithelium, in contrast to the fully labelled cell in the connective tissue.

Stained haematoxylin. Orig. mag. X320.

MIGRATION PATTERN OF CELLS.

As discussed in Section I, mitosis and migration form the two basic factors of cell function. In the body, two types of migration are recognized; active movement as occurs in leucocyte migration during phagocytosis and passive movement as seen in a tissue like skin where new cells push older cells inwards to the prickle cell layer for eventual desquamation at the surface.

In all three epithelial cell layers of the mouse gingivae, the germinative basal layers showed the initial cell labelling. It was observed that the greatest number of mitoses was occurring in both the downgrowing and oral epithelium in the tissue compartment nearest the apical end of the tooth. In the epithelial attachment there were occasional labelled cells on the surface layer of epithelium next the enamel space, even at the 1 hour interval after injection.

One of the benefits of H^3-thymidine autoradiography is that the movement pattern and life cycle of the labelled cell can be traced after division, since a fully labelled cell, when it divides, halves the labelling content of its nucleus between the two daughter cells so formed. (Messier & Leblond 1960). Since one of these cells remains in the basal layer for further division while the other migrates to the surface for desquamation, observations made of the time sequence between full labelling and complete loss of half-labelled cells from the cell population can be regarded as a measure of renewal time for the tissue. By plotting the position of labelled cells in the three areas of mouse gingivae at the various time intervals, the migration pattern of these half-labelled daughter

cells was established. For each time interval the position of the labelled cells was marked and, following the calculation of the labelling index, a composite picture of the cell migration pattern was built up. This is illustrated in Fig. 33.

One common factor which emerged was that in all epithelial areas the majority of cells were desquamated towards the coronal tip of the gingivae.

In the epithelial attachment nearest the cement enamel junction, a very rapid changeover of cells was evident. Here, the tissue was only 2-3 cells deep and a horizontal movement of cells occurred towards the enamel space to be followed by a vertical oblique movement either against the enamel surface or as a "stream" within the "attachment epithelium". It also appeared that some cells terminated their life cycle in the keratin band between the downgrowth epithelium and the epithelial attachment.

Since this experiment was completed, a report has been published by Joglekar, Toto & Gargiulo (1964) in which their findings for the interpapilla of mice support these observations. Their division of the tissue differed but the suggested migration pattern was similar in character.

It would seem that movement of cells to produce these migration patterns is passive with new cells pushing older cells towards the surface.

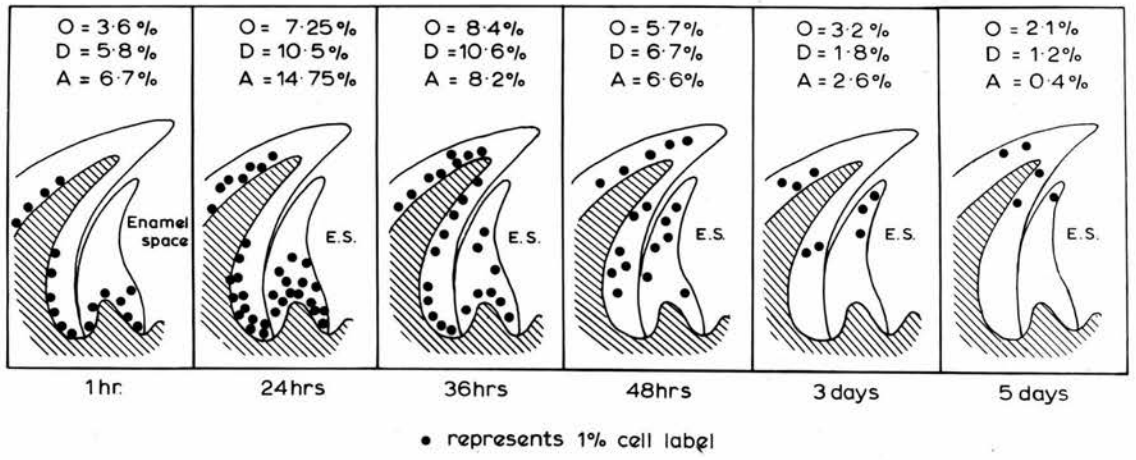

Fig. 33. Time sequence diagram of the positions of labelled cells in the retro-molar papillae of mice, assembled from observations of autoradiographs at fixed time intervals after H^3-thymidine injection.

O = Oral epithelium.

D = Downgrowth epithelium.

A = Epithelial attachment.

RENEWAL TIMES OF THE EPITHELIUM.

The turnover time or renewal time has been expressed by Leblond et al (1959) as the time taken for renewal of all the cells comprising the cell population. As shown, dividing cells which have incorporated H^3-thymidine can be followed throughout their life cycle. An argument can therefore be made that the tissue renewal time is the time taken for complete loss of the half-labelled daughter cells from the tissue compartment.

By such criteria the observation data obtained throughout this present experiment would indicate that the renewal time for the 3 areas of epithelium under examination should be expressed by the diagram in Fig. 34, viz., 24 hours for turnover of cells at the cement enamel junction of the epithelial attachment, 3-5 days for the complete turnover of the epithelial attachment and 10-12 days for the oral and downgrowth oral epithelium.

At the cement enamel junction, the epithelial layer is only some 2-3 cells thick. Consequently, the basal and surface layers are adjacent to each other. To maintain this thickness when a new cell forms in the basal layer, the surface cell requires release for desquamation. It was almost a constant finding to observe labelled cells at this part of the epithelium at the 1 hour interval after injection, whereas, at the 24 hour interval, labelling was dilute on the surface and basal layers. The remaining part of the epithelial attachment retained half-labelled cells usually for 3 days but occasionally for 5 days. The oral and downgrowth epithelium, however, did not lose all of their marked cells until 10-12 days had elapsed. Thus a difference existed in the renewal times of the 3 parts of the epithelium which constitute the mouse

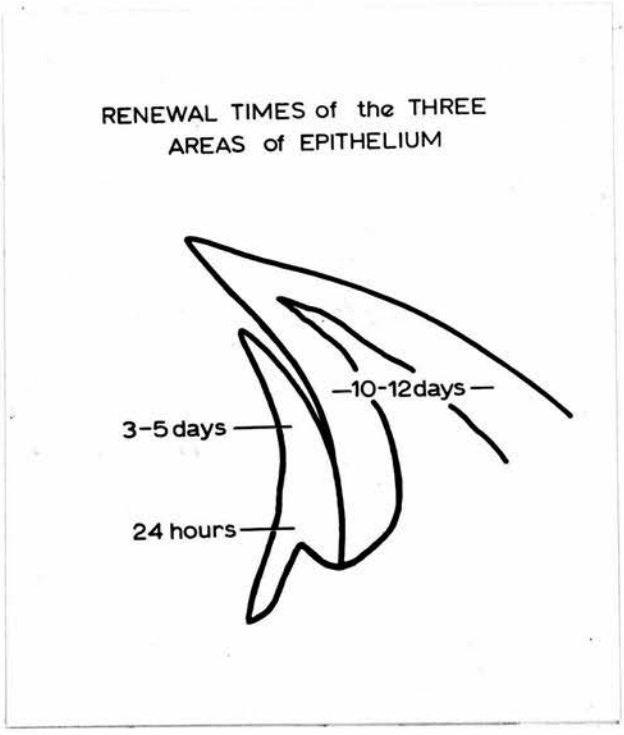

Fig. 34. Diagram giving renewal times of the epithelial attachment, downgrowth and oral epithelium, following observation of time taken for loss of half-labelled cells from these tissue compartments.

gingival papilla. This showed that attachment epithelium was renewed at twice the speed of the other two populations.

Such a method of determining renewal time is, however, open to discrepancy, since it takes no account of the variation in distance that individual cells have to migrate to the surface for desquamation. As a result of this, by the time the last half-labelled cell is desquamated, many of the cells in the tissue may have been renewed more than once. Furthermore, with increased time, there is an increased amount of mixed cell labelling in the autoradiograph which creates problems in interpretation of what constitutes a half-labelled cell and what a quarter-labelled, etcetera. A cell which is saturated with tritium from H^3-thymidine, when sectioned through its oval nucleus at right angles to its long axis, will give a reduced emission of beta rays when compared to the same cell sectioned through its long axis. Furthermore, two cells with an equal quantity of isotope incorporated within the nucleus will not give the same grain counts if placed at different levels within the section. Long exposure of autoradiographs will partly offset this result but not completely. Because of these factors it seemed that the most reliable labelling index would be that obtained from an animal sacrificed 1 hour after injection of the isotope. This would allow observations on cells before the nuclei had divided and therefore maximum emission of beta particles would be obtained and the tendency for false readings reduced.

It has been previously shown (Skougaard & Beagrie 1962) that tissue renewal can be calculated by the following formula :-

$$t = \frac{100}{r} \times \frac{S}{24}$$

t = time in days

S = DNA synthesis time

r = 1 hour radio-active index.

It was decided to use this formula and calculate the tissue renewal time for all epithelial populations so that the results obtained by the observation method could be subjected to a more critical analysis. Before making such a calculation however, it was necessary to carry out two additional experiments :-

(i) To determine the diurnal variation of the 1 hour labelling index of gingival epithelium.

(ii) To calculate the DNA synthesis time for mouse gingival epithelial cells.

DIURNAL VARIATION OF THE LABELLING INDEX.

As the 1 hour labelling index for the calculation of renewal time is an important base line, it was necessary to determine to what extent the diurnal variation would influence this index in the epithelia under examination. Such an index fluctuation for other mouse epithelial tissues has been shown by Messier & Leblond (1960).

PRESENT INVESTIGATION

For the experiment, 12 male animals of the same inbred (J.U.) stock from the Animal Genetics Department of the University of Edinburgh were selected. They were kept under identical cage, temperature and feeding conditions for 10 days prior to the experiment to stabilize their reactions to a new environment. At the start of the experiment they were 8 weeks ($\pm$ 1 day) old. The average weight of the animals was 25 grams. Divided into four groups of three, each animal was injected intramuscularly in the left hind leg with H^3-thymidine (specific activity 3 curies/millimole) at a dose of 1 micro-curie per gram body weight 1 hour before sacrifice. Times of injection for the groups were 10 a.m., 4 p.m., 10 p.m. and 4 a.m. respectively. All group injections were completed within five minutes. One hour after injection the animals were sacrificed and their vascular systems perfused via the right atrium of the heart with 10% neutral formalin, after which their heads were removed, sagitally divided and fixed in 10% formalin for 48 hours. Decalcified in a 5% solution of ethylenediamine tetra-acetic acid at room temperature, the half-head specimens were double embedded in celloidin and paraffin wax; sections were cut sagitally at 5 microns and autoradiographs then made of the gingival

tissues around the molar teeth. Pelc (1947) stripping film technique was used for this purpose and the film was exposed for 21 days in light tight boxes at 5°C. After development, the autoradiographs were stained with haematoxylin and counts were then made of the labelled and non-labelled cells of the 3 epithelial areas. Random sections were examined from each animal of the retromolar papilla of the 3rd molar tooth and at least 1,000 cells were counted for each part. A calculation was then made of the 1 hour labelled cell percentage. Examination was carried out at a magnification of 640 diameters under an oil immersion lens. Cells with 5 grains and more were considered labelled and in this way an allowance for false labelling through "background" was made to give a more accurate set of figures.

RESULTS

The results for each animal and each tissue compartment are graphically illustrated in Figs. 35, 36 and 37, whilst the average readings for the tissue are shown in Fig. 38. It can be seen that a rhythmic variation exists throughout the 24 hour period of the experiment in all cell populations.

Oral Epithelium.

The average labelling indices for the four time intervals under review were as shown in Table VI. The lowest index was at 5 a.m., the highest at 5 p.m. and the tissue showed the greatest fluctuation of the three cell populations under examination. New cells were formed in most parts of the basal layer. For the complete 24 hour period the oral epithelium had an average index of 3.53% (Table VII) which was the lowest of the three areas of

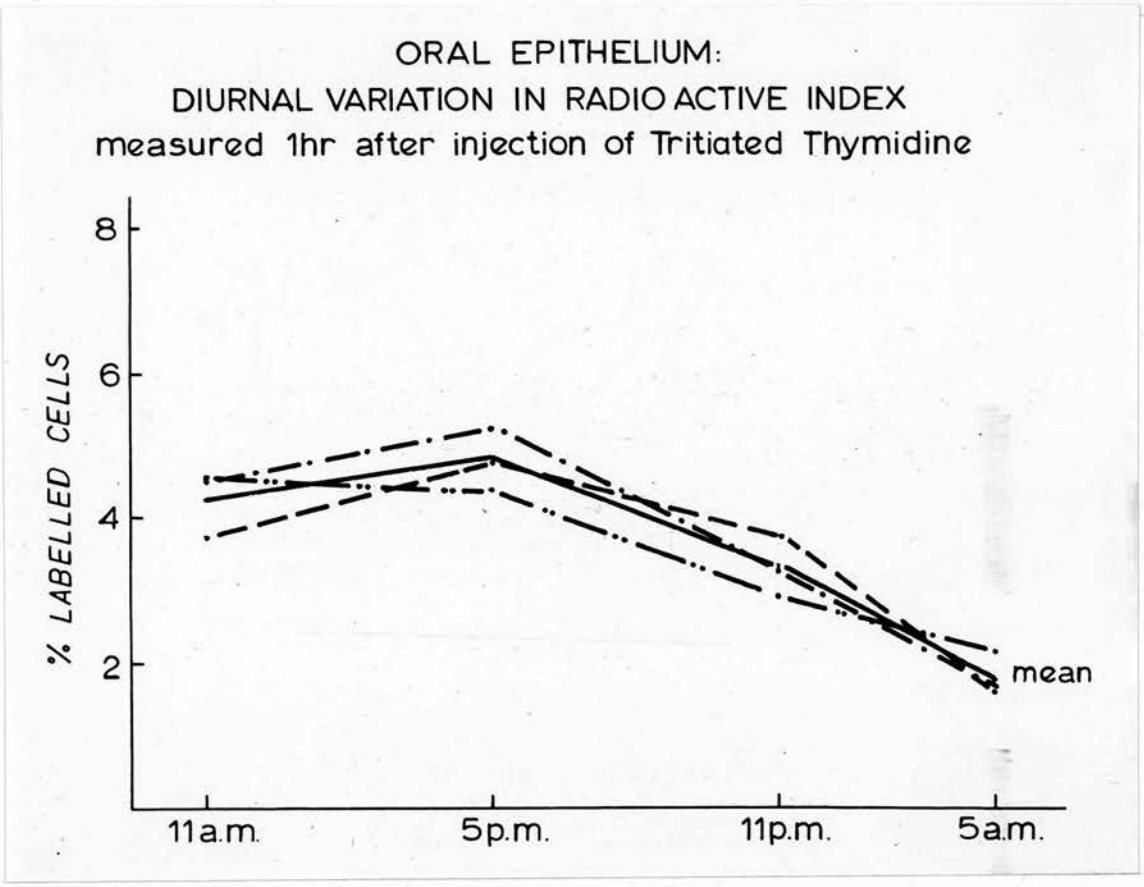

Fig. 35. Distribution and mean for oral epithelium of the radioactive indices (% labelled cells) at four time intervals with 3 animals each interval. Total of 12 readings - complete data in Appendix.

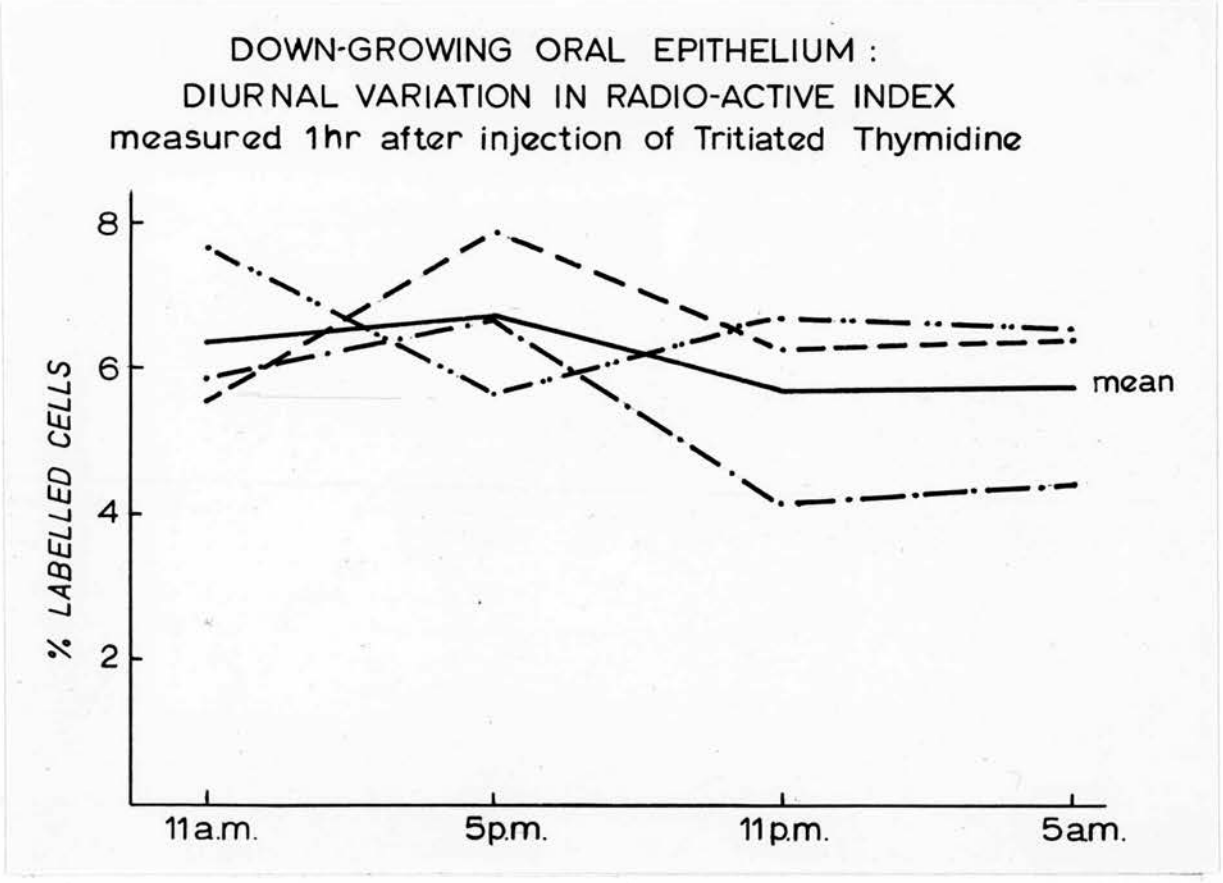

Fig. 36. Distribution and mean of the radioactive indices for down-growing oral epithelium at four time intervals - 3 animals each interval. Complete data in Appendix.

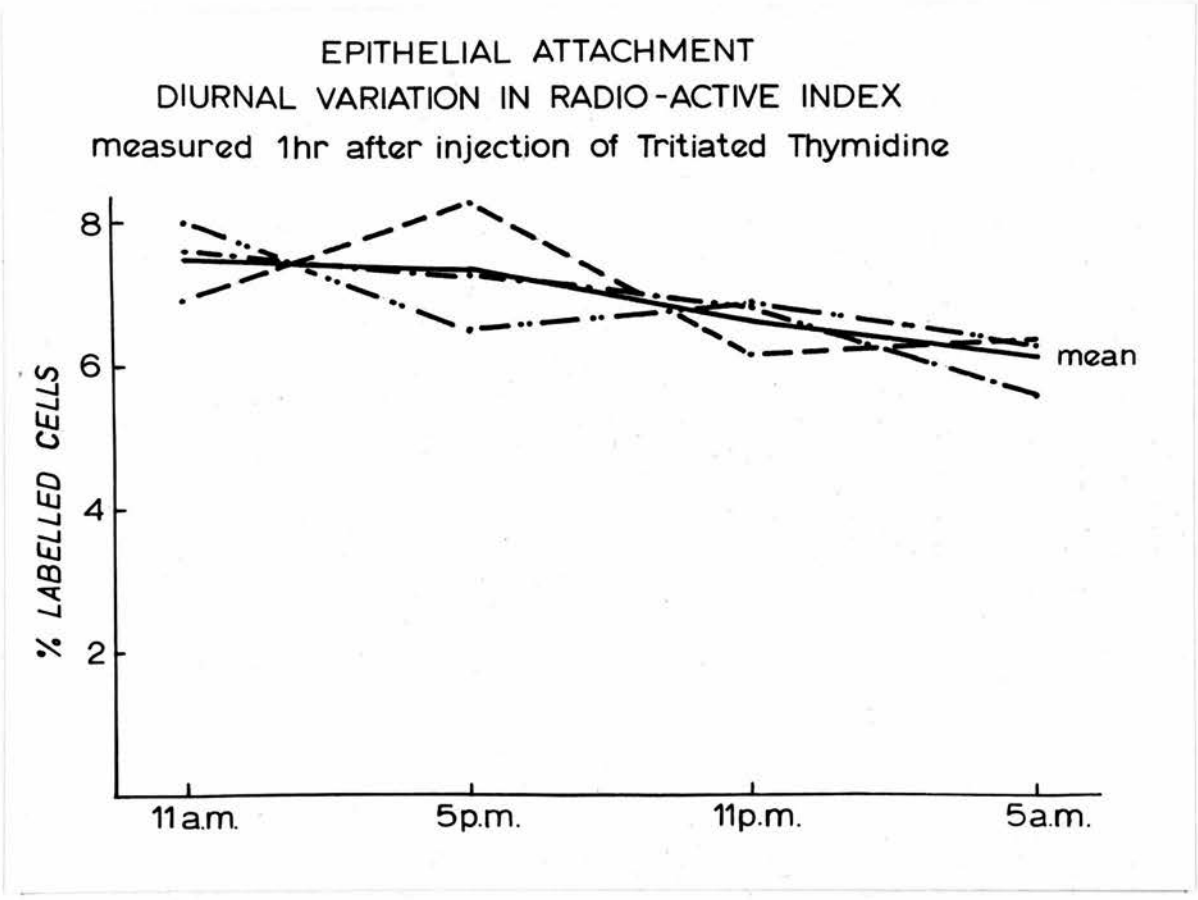

Fig. 37. Epithelial attachment distribution and mean value of the radioactive indices at four time intervals - 3 animals each interval. Complete data in Appendix.

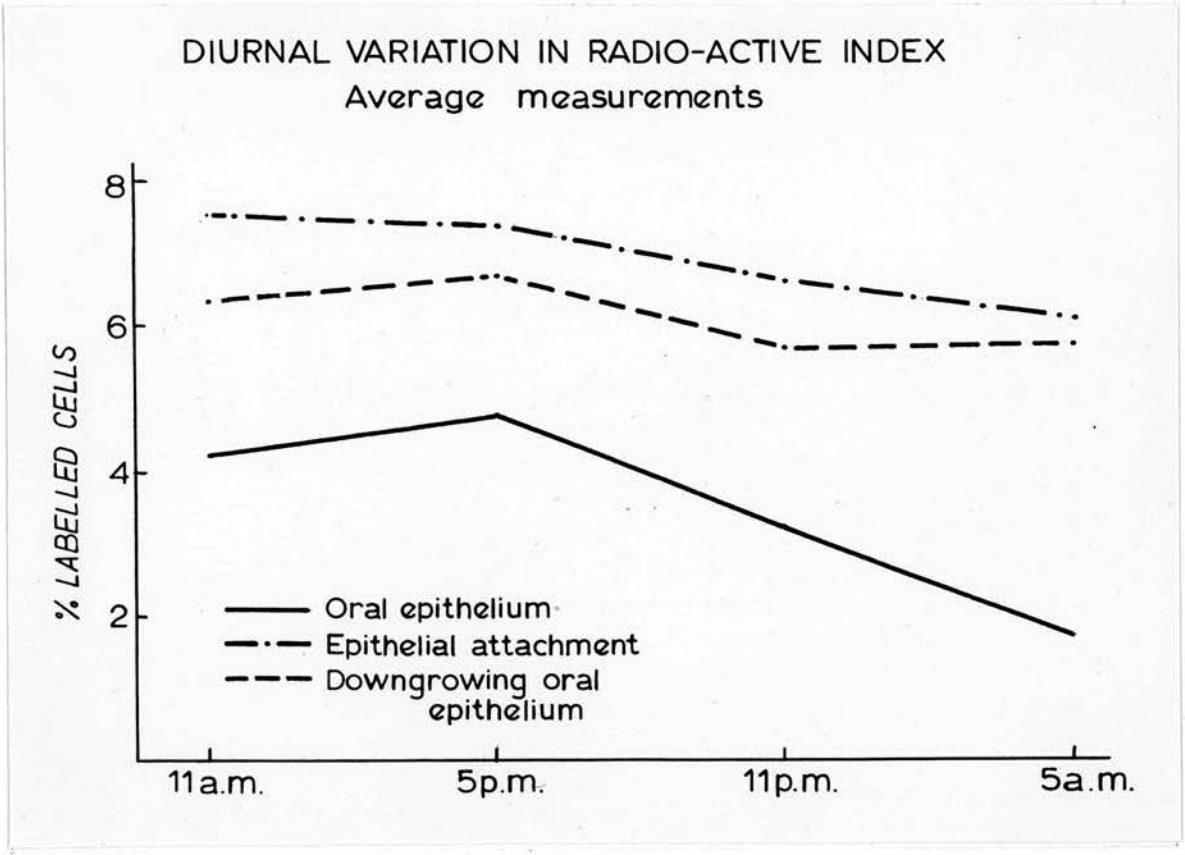

Fig. 38. The means of the radioactive indices for the three epithelial cell populations comprising the gingival tissue of mice. These are plotted against 4 time intervals throughout the day and night and demonstrate the diurnal variation.

gingival epithelium.

Downgrowth Epithelium.

In this compartment the majority of new cells were found in the tissue nearest the root of the tooth and farthest from the oral cavity. The fluctuation pattern of the lowest and highest indices was similar to that seen in the oral epithelium but it was not so marked. (Table VI : Figs. 36 & 38).

Epithelial Attachment.

New cells in this area were again mainly related to the deepest part of the tissue, i.e., next the cement enamel junction of the tooth where some labelled cells were observed adjacent to the enamel space. The majority of labelled cells were, however, in the basal layer with the lowest average index of 6.11% being registered at 5 a.m. (Table VI). The epithelial attachment area had the highest overall average for the 24 hour cycle of 6.91%. (Table VII).

TABLE VI

Average labelling index, 1 hour after injection of H^3-thymidine, in the three areas of the mouse gingivae at 4 time intervals throughout the 24 hour cycle. (3 animals each interval)

Time	Oral Epithelium	Downgrowth Epithelium	Attachment Epithelium
11 a.m.	4.26	6.35	7.52
5 p.m.	4.80	6.70	7.39
11 p.m.	3.29	5.70	6.62
5 a.m.	1.77	5.79	6.11

Total count of 3,000 cells in each area.
(Complete data in Appendix II).

TABLE VII

The average labelling index throughout 24 hour cycle, 1 hour after injection of H^3-thymidine, in the three areas of mouse gingivae.

Oral Epithelium	Downgrowth Epithelium	Attachment Epithelium
3.53	6.14	6.91

Total count of 12,000 cells in each area.
(Complete data in Appendix II).

DNA SYNTHESIS TIME.

It has previously been shown (Howard & Pelc 1953) that a proliferating cell passes through definite phases when it is preparing for mitosis :-

(G1) - post-mitotic gap or pre-synthetic phase;

(S) - DNA synthetic phase;

(G2) - pre-mitotic or post-synthetic phase;

(M) - mitosis.

This is a strict unbroken order so that injected H^3-thymidine can only be taken into the cell for DNA incorporation during the 'S' phase. If, after injection of H^3-thymidine, experimental animals are sacrificed at different time intervals, the 'S' phase time can be calculated by observing how long it takes for mitoses to become labelled. Animals which are sacrificed a short time after injection will have unlabelled mitoses as these cells will have been in a non-synthetic phase 'G2' or early mitosis. With an increased time interval, however, the mitoses will show labelling as their parent cells have been in 'S' phase at the injection time. Finally, the interval will become great enough to allow mitoses to re-appear without label from cells which, at injection, would have been in the post-mitotic gap (G1). Quastler (1960) used this method to calculate the phase lengths for mouse intestinal epithelium. Since the calculation of renewal time for gingival epithelium, using the 1 hour labelling index, also requires the DNA synthesis time of gingival cells, the following experiment was carried out.

PRESENT INVESTIGATION

In this experiment 10 male (J.U.) inbred strain mice (Animal Genetics Department, Edinburgh University) were used. As before, they were kept under identical conditions and were stabilized to their new environment for 10 days before the experiment began. When 8 weeks ($\pm$ 1 day) old with an average weight of 25 grams, they were each injected intramuscularly with tritiated thymidine (specific activity 3 curies/milli-mole) at a dosage of 1 micro-curie per gram body weight. The animals were then sacrificed at intervals of $\frac{1}{4}$ hour, $\frac{3}{4}$ hour, 1 hour, 2 hours, 4 hours, 6 hours, $7\frac{1}{2}$ hours, 8 hours, 9 hours and 10 hours after injection.

On removal, the heads were divided sagitally and fixed in 10% formal saline. Decalcified in a 5% solution of ethylenediamine tetra-acetic acid the tissues were then processed and double embedded in celloidin and paraffin wax. Sections were cut at 5 microns and covered with Kodak AR.10 stripping film according to Pelc (1947) and stored in light tight boxes at 5°C for 10 days. The autoradiographs were then developed, fixed and stained in haematoxylin.

RESULTS

50 gingival mitotic figures were examined from each animal for evidence of labelling and from this, the percentage of labelled mitoses was provided. Routine examination of each mitotic figure was made at a magnification of 640 diameters after which a more extensive examination was made at 1600 diameters.

A labelled mitosis was recognized with an average of 6-8

silver grains over each half nucleus. Such a small number of grains provided full observation of the morphological detail of the cell but at the same time the grain number was large enough to avoid making false readings through "background" fogging.

The results of the experiment are given in Table VIII. These would seem to indicate a DNA synthesis time of 7 hours since 92% of the mitoses were labelled at the 2 hour interval and 72% unlabelled at 9 hours.

TABLE VIII

The percentage of labelled mitoses in gingival epithelium of mice at various time intervals after H^3-thymidine injection.

Hours after Injection	Percentage labelled mitoses
$\frac{1}{4}$	None
$\frac{3}{4}$	None
1	18
2	92
4	98
6	95
$7\frac{1}{2}$	93
8	90
9	28
10	4

CALCULATION OF TISSUE RENEWAL TIME

As shown earlier, the tissue renewal time can be expressed by the following formula (Skougaard & Beagrie 1962) :-

$$t = \frac{100}{r} \times \frac{S}{24}$$

where t = renewal time in days

S = DNA synthesis time in hours

r = the 1 hour radioactive index expressed as the percentage of labelled cells.

By applying the DNA synthesis time and the average 1 hour labelling indices from Table VII, the following turnover time for the three areas of mouse gingival epithelium are given :-

Oral Epithelium = $\frac{100}{3.53} \times \frac{7}{24}$ = 8.26 days

Downgrowth Oral Epithelium = $\frac{100}{6.14} \times \frac{7}{24}$ = 4.75 days

Epithelial Attachment = $\frac{100}{6.91} \times \frac{7}{24}$ = 4.15 days

However, as discussed in Section I, following an injection of H^3-thymidine at this dosage and concentration, mathematical calculations, using the 1 hour labelling indices, require to take cognizance of a 16% increase in mitosis. Thus the 1 hour average labelling indices for Table VII should read - 2.97, 5.16 and 5.80. The corrected figures for renewal times would then be :-

Oral Epithelium - 9.82 days = 10 days

Downgrowth Oral Epithelium - 5.65 days = $5\frac{1}{2}$ days

Epithelial Attachment - 5.03 days = 5 days.

DISCUSSION.

It would appear, both by the observation method and by calculation from the 1 hour labelling index, that the epithelium next the enamel of the tooth is renewed at twice the speed of the oral part of the gingival papilla. By both methods the renewal times for oral and epithelial attachment cell populations are in close agreement. On the other hand, the downgrowth epithelium has a renewal time by the observation method which is double the result arrived at by calculation through the 1 hour index. This is explained by the fact that cells in this tissue compartment, like those of oral epithelium, are retained for some time in the keratin layer before being lost from the surface, thus allowing an extended observation of the half-labelled cells. Furthermore, in this cell population, labelled cells have a considerable distance to move from the main progenitor site at the apical end of the downgrowth epithelium to the coronal tip of the papilla.

A renewal time of 2-4 days for the gingivae of mice has been calculated by Meyer zum Gottesberge & Koburg (1963) which is in reasonable agreement with the calculated renewal times for the epithelial attachment and downgrowing epithelium. Use was made of H^3-thymidine in their study but the gingival tissue was not divided in the same way as reported here.

For the marmoset (Skougaard & Beagrie 1962), oral epithelium was renewed in 10 days and attachment epithelium in 6 days and these times closely resemble the findings of this present investigation.

Trott & Gorenstein (1963) reported from a colchicine study on rat gingivae that the renewal time of oral epithelium was 44 days, the epithelial attachment 11 days and the downgrowth oral epithelium 16 days. Although the ratios of the population renewal times were similar, the actual numerical results were grossly at variance with those reported here. It is highly unlikely that such a variance can be explained by use of different experimental animals since both were rodents. It is probable that the difference in technique between the studies is the more tenable cause, particularly as colchicine is a tissue poison.

The DNA synthesis time of 7 hours for mouse gingival epithelial cells in this study is 3 hours less than that reported by Toto & Ojha (1962) who gave an estimated time of 10 hours for tongue epithelium of the mouse. However the results do not differ greatly from Rubini et al (1962) who suggested an average of 6 hours. Koburg & Maurer (1962) and Cameron & Greulich (1963) were of the opinion that a constant of 6-8 hours was the 'S' phase time range for all mammalian cells. Indeed, Cameron & Greulich (1963), using tongue and cheek epithelium of the mouse as the test tissues, suggested an 'S' phase of 6.9 - 7.2 hours for oral epithelium and this corresponds with the figures claimed for this present investigation.

Turning to diurnal variation of mitoses in oral epithelium, Mühlemann et al (1959) and Trott & Gorenstein (1963) using the rat as the experimental animal, both found greatest mitotic activity at 7 a.m. and lowest at 7 p.m. Cooper & Franklin (1940) and Blumenfeld (1943), reporting on mouse epidermis mitoses, recorded

the highest figure between 8-10 a.m. and the lowest between 8-10 p.m. All of these workers, however, were reporting on indices from histological observations of mitotic figures, whereas, in this study, the results are not of actual mitoses, but of cells in the DNA synthesis phase of the mitotic cycle, that is, preparing for division. It is to be expected then, that a time differential should be reported in the order of 12 hours as the labelled cells would have to complete both their synthesis and pre-mitotic phases before entering mitosis. The differences in the labelling indices at the four time intervals of the day and night nevertheless demonstrate that a diurnal variation in these tissues is present. It is thus essential in experiments using the labelling index of gingival tissues to take cognizance of the diurnal variance of the tissues and in calculations involving short time intervals following H^3-thymidine injection to determine the 24 hour average.

The epithelial attachment had the highest mitotic activity of the three cell populations examined - a fact which was true at all time intervals, day and night. In this tissue, the greatest number of labelled cells was found nearest the cement enamel junction similar to the findings reported in Section II relating to the newly erupted tooth. Such a result gives support to the contention of Scott & Symons (1961) and Sicher (1962) that after oral epithelium and remnants of the dental epithelium blend with eruption of the teeth, the dental epithelial remnant retains greater growth potential for extension on to the cementum of the root. Growth of epithelium on to the cementum is a feature of periodontal disease and tissue aging and the continued presence of cells ready for

mitosis at the cement enamel junction is seen as a protective mechanism. The higher labelling indices for the epithelial attachment compared with the downgrowth epithelium indicate that the downgrowth has a lower growth potential than the attachment epithelium. The main progenitor site for new cells in the downgrowth epithelium is also found at the most apical part. A feature of gingivitis is the presence of finger-like extensions of epithelium from these superficial tissues and the presence of this progenitor site would indicate the "cell bed" for such growth.

All three epithelial cell areas of the mouse gingivae have been shown to have an active renewing cell population. Cells were lost via the gingival crevice and also via the keratin band between the downgrowth epithelium and epithelial attachment. It is difficult to assess whether the cells of the epithelial attachment contribute to the keratin formation but it seems that the majority of cells in all compartments are lost finally at the coronal tip of the gingivae. Thus the migration of cells is horizontal and oblique. Since this movement is traced in all three epithelial areas, it is suggested that the shape of the tissue in part determines the direction of migration. Examination of autoradiographs of tongues from these same animals supports such a conjecture since a similar migration pattern of cells is seen around the tongue papillae.

The epithelial attachment, having an active proliferating cell population and the shortest renewal time of all areas, would therefore not appear to be a degenerative tissue as claimed by other workers - Becks (1929) : Skillen (1930a: 1930b) and Hirt, Hartl & Mühlemann (1955).

The greater mitotic activity in this area is probably an adaption to the function of the tissue. Certainly, with such a rapid cell turnover, foreign material would have difficulty in penetrating the potential space between the gingivae and the tooth crown because of the outward flow of detached cells. It is difficult to visualize in these circumstances, where loose cells are moving on the surface, how an attachment of epithelium of the type suggested by Gottlieb (1921) can occur.

Instead, this present work would add support to the contention of Waerhaug (1952) and McHugh (1959) that the gingival tissue around the molar teeth of mice is present as a "cuff" and that a microscopic space exists between the enamel of the tooth and the epithelium lining the gingivae.

CONCLUSIONS.

In the mature gingival tissue around the erupted molar teeth of the mouse :-

(1) All cell populations of the epithelium were of the renewing type.

(2) The migration pattern of cells had a horizontal and oblique direction and was probably of a passive type.

(3) The labelling index of each tissue cell population showed a diurnal variation with the lowest indices between 4-5 in the morning and the highest between 4-5 in the evening.

(4) The oral epithelial tissue compartment of the gingivae showed the greatest diurnal variation.

(5) The epithelial attachment area had the highest 1 hour labelling index and therefore the greatest growth potential.

(6) The progenitor sites for both downgrowing oral epithelium and epithelial attachment occurred at the most apical part of the tissue compartment whilst oral epithelium did not appear to have any definite pattern.

(7) For gingival epithelial cells, the DNA synthesis phase (S) was calculated as 7 hours.

(8) The renewal times for the three epithelial populations under study were :-

10 - 12 days for oral epithelium

10 - 12 days for downgrowing oral epithelium

3 - 5 days for epithelial attachment

by observing half-labelled cells through the tissue to desquamation, and

10 days for oral epithelium

$5\frac{1}{2}$ days for downgrowing oral epithelium

5 days for epithelial attachment

by calculation using the 1 hour labelling index and DNA synthesis time.

(9) A microscopic space must exist between the lining epithelium of the mouse gingivae and the enamel, thus the term "epithelial cuff" rather than "epithelial attachment" is required to describe the relationship between the tooth and the gingival epithelium.

GENERAL CONCLUSIONS AND THEIR SIGNIFICANCE FOR FUTURE STUDY.

Concerned with the observation and study of mitoses in the gingival epithelium of mice, this work has been divided into three sections.

Because of the varied opinions regarding the effects of tritiated thymidine on the cell systems, an experiment was described and discussed in which the action of tritiated thymidine on cell mitoses was tested on oral epithelial cells. From this data it was concluded that either an injection of plain thymidine or tritiated thymidine will raise the mitotic index of oral epithelial tissues. The test tissues were tongue and palate epithelium and for tritiated thymidine at a dosage of 1 micro-curie per gram body weight of animal, the mitoses increase was 6.5% - 25.5%, while plain thymidine produced an increase in mitoses of 22.2% - 37.2%. The use of tritiated thymidine and autoradiography was therefore shown to be highly suitable for studying the histogenesis of gingival epithelium and for the examination of the life cycle of cells of the mature gingivae around the erupted teeth.

Prior to amelogenesis, it was evident from H^3-thymidine labelling and cell migration patterns that the stratum intermedium layer of the enamel organ provided the cells of the stellate reticulum. After a period of no mitotic activity the stratum intermedium then became the cell layer which produced the mitotic proliferation for expansion of the reduced enamel epithelium. When the tooth approached the oral cavity, many mitotic labelled cells were found in the stratum intermedium. With the advance

of this active proliferating layer, the oral epithelium lost the rete pegs and exhibited fewer mitoses. These observations thus indicate that the major contribution through epithelial cell mitosis for the formation of gingival tissue is made by the reduced enamel epithelium in general and the stratum intermedium layer in particular and that this joins with a downgrowing layer of oral epithelium to form the mature tissue.

The stimulative mechanism for this sudden proliferation of the reduced enamel epithelium is not explained but it was noted that the connective tissues over the erupting tooth exhibited little or no mitoses and had poor staining characteristics. Aiseinberg & Aiseinberg (1951) : Baume (1952) : Hunt & Paynter (1959 : 1963) all observed increased epithelial proliferation in areas where connective tissue dissolution had taken place and the autoradiographic observations in this present study showing a lack of mitoses in this area would support such a contention.

During eruption of the tooth the ameloblasts showed no labelling to indicate DNA synthesis - a finding in this layer of cells from the end of enamel matrix production. As the tooth erupted with ameloblasts contacting the enamel surface, an organic connection by the Tomes' processes to the enamel would be present. Such an organic attachment would maintain the integrity of the gingival epithelium until the ameloblasts were replaced by squamous cells through a normal replacement mechanism and passive cell movement.

When the gingivae were mature, mitotic activity in the epithelium next the enamel was then greater than the oral and downgrowing parts and replacement of cells took place every 5 days. Thus, no support can be given to the attachment theory of Gottlieb (1921). Instead, it would seem more likely that an adhering cell mechanism to the tooth surface would be present, certainly as far as the mouse is concerned. Recently, Toto & Sicher (1964) have suggested that an adhesion of the epithelium to the tooth enamel occurs through the medium of a mucopolysaccharide elaborated by the epithelial cells and although not examined in this study, such a theory would seem satisfactory.

The rapid replacement time of "attachment" gingival epithelium probably acts as a passive defence mechanism since the continued loss of cells to the oral cavity from this potential space would help to prevent bacteria gaining access to the deeper epithelial structures. Since this epithelium is devoid of keratin, some such mechanism would be necessary. Brill (1962) suggested that fluid transudate with antibodies added to the defence of this area and Cowley (1965) has shown by fluorescent antibody studies the presence of such a mechanism.

One of the histopathological features of advancing periodontal disease is the presence of an epithelial extension on to the surface of the cementum of the tooth root. In this study, labelled cells were always present in epithelium at the cement enamel junction and it would seem that as soon as any lysis of connective tissue fibres occurred at this part, these labelled cells, on division, would be in a position to migrate over the connective tissue breach. Thus

the epithelium of the gingivae through mitoses, cell migration and desquamation, has a constant role to play in maintaining the health of the superficial periodontal tissues.

The effects of hormones and vitamins on the gingival epithelium require further investigation. Oestrogens, for example, directly and indirectly affect the gingival tissues, whilst Vitamin A is related to the mechanism of epithelial keratinization. The presence of adjacent keratinized and non-keratinized epithelium in the gingivae of mice should provide an excellent model for studying the biological effects of these substances. Where do the effects occur? Is there a decreased or increased mitotic index? Is there a delayed DNA synthesis? These are but a few questions requiring answer by future study.

APPENDIX

GROUP 1

In 24 mice, the percentage of mitoses in tongue epithelium together with the mean ($\bar{x}$) and variance (S^2), 3 hours after injection of Saline.

Animal	% Mitoses
1S	0.99
2S	1.13
3S	0.62
4S	1.11
5S	0.85
6S	1.17
7S	1.28
8S	1.11
9S	1.23
10S	1.14
11S	1.22
12S	1.16
13S	0.60
14S	1.00
15S	1.00
16S	1.32
17S	1.10
18S	0.90
19S	1.40
20S	1.00
21S	0.90
22S	0.79
23S	1.43
24S	1.40
	25.85

$$\sum x^2 = 29.003$$

$$\bar{x} = 1.077$$

$$S^2 = 0.0505$$

GROUP 1

In 24 mice, the Arc Sin transformations of the percentage mitoses in tongue epithelium together with the mean (x_1), 3 hours after injection of Saline.

Animal	Arc Sin Transformations
1S	5.71
2S	6.02
3S	4.52
4S	6.02
5S	5.29
6S	6.29
7S	6.55
8S	6.02
9S	6.29
10S	6.02
11S	6.29
12S	6.29
13S	4.44
14S	5.74
15S	5.74
16S	6.55
17S	6.02
18S	5.44
19S	6.80
20S	5.74
21S	5.44
22S	5.10
23S	6.80
24S	6.80
	141.92

$$\sum x^2 = 848.76$$

$$\bar{x}_1 = 5.913$$

GROUP 2

In 24 mice, the percentage of mitoses in tongue epithelium together with the mean ($\bar{x}$) and variance (s^2), 3 hours after injection of Thymidine.

Animal	% Mitoses
1T	1.80
2T	1.00
3T	0.92
4T	1.18
5T	1.41
6T	1.07
7T	0.94
8T	1.20
9T	1.35
10T	1.31
11T	1.80
12T	1.24
13T	1.35
14T	1.04
15T	1.65
16T	1.11
17T	1.52
18T	1.29
19T	1.22
20T	1.44
21T	1.33
22T	1.94
23T	1.33
24T	1.25
	31.69

$$\sum x^2 = 43.5107$$

$$\bar{x} = 1.3204$$

$$s^2 = 0.0721$$

GROUP 2

In 24 mice, the Arc Sin transformations of the percentage mitoses in tongue epithelium together with the mean ($\bar{x}_1$), 3 hours after injection of Thymidine.

Animal	Arc Sin Transformations
1T	7.71
2T	5.74
3T	5.50
4T	6.29
5T	6.80
6T	6.02
7T	5.56
8T	6.29
9T	6.80
10T	6.55
11T	7.71
12T	6.29
13T	6.80
14T	5.74
15T	7.49
16T	6.02
17T	7.04
18T	6.55
19T	6.29
20T	6.80
21T	6.55
22T	7.92
23T	6.55
24T	6.55
	157.56

$$\sum x^2 = 1044.545$$

$$\bar{x}_1 = 6.565$$

GROUP 3

In 24 mice, the percentage of mitoses in tongue epithelium together with the mean ($\bar{x}$) and variance (S^2), 3 hours after injection of H^3-thymidine.

Animal	% Mitoses
1H	0.93
2H	1.12
3H	0.99
4H	1.11
5H	1.44
6H	1.09
7H	1.06
8H	0.87
9H	1.29
10H	1.09
11H	1.24
12H	1.11
13H	0.95
14H	1.09
15H	1.06
16H	1.11
17H	1.37
18H	1.15
19H	1.49
20H	1.31
21H	0.96
22H	1.20
23H	0.88
24H	1.66
	27.57

$\sum x^2 = 32.569$

$\bar{x} = 1.1487$

$S^2 = 0.0391$

GROUP 3

In 24 mice, the Arc Sin transformations of the percentage mitoses in tongue epithelium together with the mean ($\bar{x}_1$), 3 hours after injection of H^3-thymidine.

Animal	Arc Sin Transformations
1H	5.53
2H	6.02
3H	5.71
4H	6.02
5H	6.80
6H	6.02
7H	6.02
8H	5.35
9H	6.55
10H	6.02
11H	6.29
12H	6.02
13H	5.59
14H	6.02
15H	6.02
16H	6.02
17H	6.80
18H	6.29
19H	7.04
20H	6.55
21H	5.62
22H	6.29
23H	5.38
24H	7.49
	147.46

$$\sum x^2 = 912.387$$

$$\bar{x}_1 = 6.14$$

GROUP 1

In 24 mice, the percentage of mitoses in palate epithelium together with the mean ($\bar{x}$) and variance (s^2), 3 hours after injection of Saline.

Animal	% Mitoses
1S	0.91
2S	0.58
3S	0.55
4S	0.90
5S	0.46
6S	1.36
7S	0.58
8S	0.56
9S	0.86
10S	0.77
11S	1.09
12S	1.03
13S	1.53
14S	1.29
15S	1.11
16S	1.36
17S	1.22
18S	0.83
19S	0.78
20S	0.61
21S	1.35
22S	0.81
23S	1.07
24S	0.88
	22.49

$$\sum x^2 = 23.212$$

$$\bar{x} = 0.937$$

$$s^2 = 0.093$$

GROUP 1

In 24 mice, the Arc Sin transformations of the percentage mitoses in palate epithelium together with the mean ($\bar{x}_1$), 3 hours after injection of Saline.

Animal	Arc Sin Transformations
1S	5.47
2S	4.37
3S	4.25
4S	5.44
5S	3.89
6S	6.80
7S	4.37
8S	4.29
9S	5.32
10S	5.03
11S	6.02
12S	5.74
13S	7.04
14S	6.55
15S	6.02
16S	6.80
17S	6.29
18S	5.23
19S	5.07
20S	4.48
21S	6.55
22S	5.16
23S	6.02
24S	5.38
	131.58

$$\sum x^2 = 740.688$$

$$\bar{x}_1 = 5.483$$

GROUP 2

In 24 mice, the percentage of mitoses in palate epithelium together with the mean ($\bar{x}$) and variance (S^2), 3 hours after injection of Thymidine.

Animal	% Mitoses
1T	1.13
2T	1.19
3T	1.03
4T	0.82
5T	1.20
6T	1.12
7T	1.20
8T	1.16
9T	0.86
10T	1.38
11T	1.30
12T	1.20
13T	1.68
14T	1.00
15T	1.24
16T	1.94
17T	1.09
18T	1.34
19T	1.52
20T	1.15
21T	1.69
22T	1.44
23T	1.92
24T	1.37
	30.97

$$\sum x^2 = 41.9135$$

$$\bar{x} = 1.290$$

$$S^2 = 0.0853$$

GROUP 2

In 24 mice, the Arc Sin transformations of the percentage mitoses in palate epithelium together with the mean ($\bar{x}_1$), 3 hours after injection of Thymidine.

Animal	Arc Sin Transformations
1T	6.02
2T	6.29
3T	5.74
4T	5.20
5T	6.29
6T	6.02
7T	6.29
8T	6.29
9T	5.32
10T	6.80
11T	6.55
12T	6.29
13T	7.49
14T	5.74
15T	6.29
16T	7.92
17T	6.02
18T	6.55
19T	7.04
20T	6.29
21T	7.49
22T	6.80
23T	7.92
24T	6.80
	155.45

$$\sum x^2 = 1018.647$$

$$\bar{x}_1 = 6.477$$

GROUP 3

In 24 mice, the percentage of mitoses in palate epithelium together with the mean ($\bar{x}$) and variance (S^2), 3 hours after injection of H^3-thymidine.

Animal	% Mitoses
1H	0.89
2H	1.22
3H	1.20
4H	0.76
5H	0.96
6H	0.98
7H	0.70
8H	0.85
9H	0.83
10H	1.42
11H	1.51
12H	0.96
13H	1.55
14H	1.14
15H	1.54
16H	1.54
17H	1.26
18H	1.20
19H	1.59
20H	1.17
21H	1.52
22H	1.40
23H	1.20
24H	0.85
	28.24

$$\sum x^2 = 35.1024$$

$$\bar{x} = 1.176$$

$$S^2 = 0.822$$

GROUP 3

In 24 mice, the Arc Sin transformations of the percentage mitoses in palate epithelium together with the mean ($\bar{x}_1$), 3 hours after injection of H^3-thymidine.

Animal	Arc Sin Transformations
1H	5.41
2H	6.29
3H	6.29
4H	5.00
5H	5.62
6H	5.68
7H	4.80
8H	5.29
9H	5.23
10H	6.80
11H	7.04
12H	5.62
13H	7.04
14H	6.02
15H	7.04
16H	7.04
17H	6.55
18H	6.29
19H	7.27
20H	6.29
21H	7.04
22H	6.80
23H	6.29
24H	5.29
	148.03

$$\sum x^2 = 926.16$$

$$\bar{x}_1 = 6.1679$$

APPENDIX II

The individual and average labelling indices for mouse gingival epithelium at four intervals throughout the 24 hour cycle - 3 animals each interval - 1 hour after injection of H^3-thymidine. (Counts of 1,000 cells each epithelial cell population).

		1 hour Tissue Labelling Index (Percentage Labelled Cells)		
Animal No.	Sacrifice Time	Oral Epithelium	Downgrowth Epithelium	Attachment Epithelium
1	11 a.m.	3.76	5.55	6.92
2	11 a.m.	4.50	5.86	7.63
3	11 a.m.	4.52	7.65	8.00
	Average	4.26	6.35	7.52
4	5 p.m.	4.77	7.87	6.54
5	5 p.m.	4.39	6.62	7.30
6	5 p.m.	5.25	5.63	8.33
	Average	4.80	6.70	7.39
7	11 p.m.	3.75	6.28	6.17
8	11 p.m.	3.28	4.14	6.83
9	11 p.m.	2.83	6.68	6.88
	Average	3.29	5.70	6.62
10	5 a.m.	1.56	6.40	6.40
11	5 a.m.	1.63	4.43	5.60
12	5 a.m.	2.13	6.55	6.33
	Average	1.77	5.79	6.11

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